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

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eLife Assessment

This is an **important** study reporting a new phenotype for a gene cluster that has previously been associated with the responses of the Gram-negative opportunistic pathogen Pseudomonas aeruginosa to flow fluid. Expression of the froABCD gene cluster is induced by HOCl in vitro and by activated immune cells, which produce these types of reactive chlorine species and the evidence presented by the authors is in many places **convincing**. Overall, the authors have been responsive to the previous review, although the exact mechanism of fro-induction by HOCl remains unclear. The high cysteine- and methionine content of the anti-sigma factor FroI hints at a direct oxidative modification of this protein during activation of the operon and a corneal infection model shows that fro is upregulated in P. aeruginosa 20 h after infection, but the evidence that HOCl is the causative agent of fro upregulation under these conditions is at present circumstantial. This study is of interest to infection biologists interested in mechanisms of bacterial pathogenicity.

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Abstract

Neutrophils kill bacteria by producing an array of lethal molecules, including hypochlorous acid (HOCl). The extent to which pathogens detect HOCl from neutrophils and defend themselves appropriately has not been understood. We report that the opportunistic pathogen *Pseudomonas aeruginosa* responds to activated neutrophils by upregulating its expression of an operon which was previously shown to be activated by fluid flow, and named the flow regulated operon (Fro). We found that *fro* is upregulated in a mouse infection model where neutrophil influx occurs, specifically by HOCl and not by other neutrophil defense factors including LL-37, histones, or H_2O_2. HOCl preferentially oxidizes methionine and cysteine residues. As the Fro operon is regulated by the anti-σ factor FroI, which contains the highest density of these residues of all anti-σ factors, this raises the possibility that FroI is an HOCl sensor. In support of this, we found that via the σ factor FroR, HOCl induces upregulation of methionine sulfoxide reductases that relieve otherwise lethal oxidative stress. These findings suggest a model in which flow transports

oxidizing molecules that activate the *fro* operon, establishing an early warning system for *P. aeruginosa* that improves its survival against host immune defenses and persistence during infection.

Impact statement

The opportunistic pathogen *Pseudomonas aeruginosa* withstands the body's first-line of defense, neutrophils, by detecting the hypochlorous acid that these cells produce and increasing their own tolerance towards it.

Introduction

The Gram-negative opportunistic pathogen *P. aeruginosa* is a major cause of hospital-acquired infections and is a significant antibiotic resistance threat. To survive in a broad range of conditions, this bacterium has evolved a variety of signaling pathways that detect changes in nutrients, chemical gradients, and flow, and activates responses that promote its survival (Fang et al., 2016; Sanfilippo et al., 2019). Understanding how extracellular-sensing pathways detect and respond to host cues is critical for improving the treatment of bacterial infections and minimizing the generation of antibiotic-resistant bacteria.

Mammalian immune systems respond to the presence of bacterial pathogens by recruiting immune cells that migrate rapidly through the circulatory system to sites of infection (Aymonnier et al., 2023). Neutrophils are the first and most abundant immune cells recruited to inflamed or damaged tissues (Lavoie et al., 2011) and are stimulated into an "activated" state by bacterial components, such as the membrane constituent lipopolysaccharide. Activated neutrophils produce multiple products that are toxic to bacteria including reactive oxygen species (ROS) and reactive chlorine species (RCS) (Hampton et al., 1998; Segal, 2005; Sultana et al., 2020; Ulfig and Leichert, 2020).

While the toxic effects of ROS on bacteria are understood, far less is known about how bacteria are affected by RCS (Fang, 2011; Gray et al., 2013). Hypochlorous acid (HOCl) is a major RCS product of neutrophils that is formed through myeloperoxidase enzymes from hydrogen peroxide (H_2O_2) and chloride ions, and is strongly bactericidal (Albrich and Hurst, 1982; Harrison and Schultz, 1976; Segal, 2005; Winterbourn et al., 2016). *P. aeruginosa* is a potent activator of neutrophil respiratory bursts (Jensen et al., 1990) but whether and how the bacteria detect and respond to neutrophil components is not understood.

P. aeruginosa responds to shear generated by the flow of fluids via the *fro* operon, which is upregulated in the bacterium during infection in humans (Cornforth et al., 2018; Padron et al., 2023; Sanfilippo et al., 2019) (Figure 1A). In addition, *fro* is necessary for successful colonization of the gastrointestinal tract and lung infections (Potvin et al., 2003; Skurnik et al., 2013), although the reasons for this is unclear. Given its important role in infection, it is possible that Fro could respond to immune-associated cues, such as the presence of neutrophils. The operon consists of *PA14_21570*, *PA14_21580*, *PA14_21590*, and *PA14_21600*, which was termed *froABCD* (Sanfilippo et al., 2019), and the extracytoplasmic function (ECF) sigma (σ) and anti-σ factors *PA14_21550* and *PA14_21560* (Boechat et al., 2013), which were termed *froR* and *froI* respectively, by (Sanfilippo et al., 2019). Expression of *froABCD* is regulated by FroR and FroI: overexpression of *froR* upregulates *fro*, whereas overexpression of *froI* downregulates *fro* (Sanfilippo et al., 2019). Flow was reported to activate FroR-dependent transcription of *froABCD* through a mechanism that involves chemical transport (Padron et al., 2023).

Here, we show that the *fro* operon responds directly to HOCl, which is produced robustly by neutrophils during immune response. Using a reporter system in live bacteria and through quantitative measurements of transcription, we show that *fro* expression is upregulated by activated neutrophils and in a murine model of corneal infection. Through transcriptional profiling, we determine that bacteria mitigate the toxic effects of HOCl through a FroR-dependent

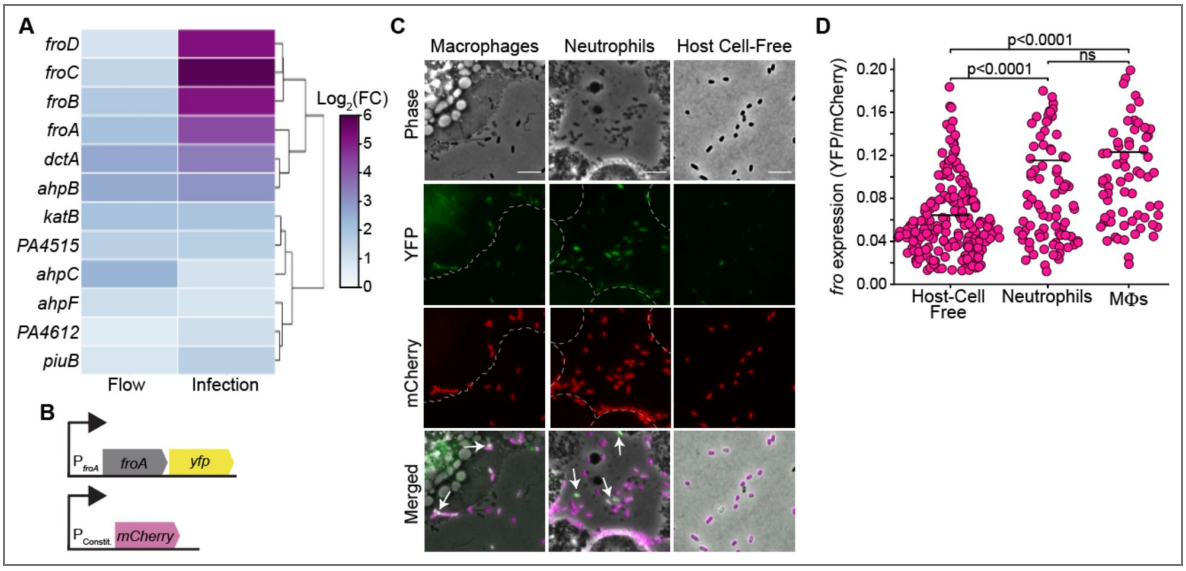


Figure 1. Activation of *fro* promoter transcription by flow and during infection of human tissue.

(A) RNA-seq heatmap of *P. aeruginosa* genes that are upregulated by at least 3-fold by flow and during infection in human wounds. Datasets are from (Cornforth et al., 2018; Sanfilippo et al., 2019). $Log_2(FC)$ indicates the log_2 of the fold-change. **(B)** Schematic of the *P. aeruginosa* fluorescent reporter strain AL143 that contains a transcriptional fusion of *yfp* to the *fro* promoter and expresses mCherry under a constitutive promoter. **(C)** Representative phase-contrast and fluorescence images and **(D)** corresponding *fro* expression as determined by the ratio of YFP to mCherry fluorescence, of *P. aeruginosa* strain AL143 co-incubated with neutrophils, macrophages (MΦs), or host cell-free medium for 30 minutes. Dashed lines in images indicate boundaries of host cells and white arrows indicate *P. aeruginosa* with intense YFP fluorescence relative to that of mCherry. Data points indicate individual *P. aeruginosa* cells from a single experiment and bars indicate the average. Images in (C) are representative of three independent experiments and scale bars represent 5 μm. P-values were obtained using Welch's t-test of data from single experiments, with values of p>0.05 denoted as ns.

mechanism that activates methionine sulfoxide reductases to relieve oxidative stress. These data suggest a novel mechanism in which bacterial defense against host innate immunity is primed by a single system that detects both flow and HOCl.

Results

Macrophages and neutrophils activate *fro* expression in *P. aeruginosa* under non-flow conditions

To investigate whether immune cells directly activate the *fro* operon, we quantified *fro* expression using a *P. aeruginosa* strain that co-expresses the yellow fluorescent protein (YFP) under the transcriptional control of the *fro* promoter and mCherry under the control of a constitutive promoter (Figure 1B). While *fro* expression increased under flow (Figure 1—figure supplement 1A), experiments in this study were performed in the absence of flow in order to deconvolve the effects of flow from potential activation of *fro* by immune cells. Because neutrophils and macrophages are the first immune cells recruited to sites of infection, we co-incubated *P. aeruginosa* with human neutrophils or mouse macrophages for 30 minutes. Relative to *P. aeruginosa* that were incubated in host cell-free media, *P. aeruginosa* co-incubated with either neutrophils or macrophages increased YFP/mCherry ratios by comparable levels (Figure 1C-D), suggesting that close proximity to or direct interaction with these host immune cells increases *fro* expression in *P. aeruginosa*.

Activated neutrophils induce *fro* expression

To assess whether *fro* upregulation in *P. aeruginosa* could be part of a response to respiratory bursts from neutrophils and macrophages, we focused on the *fro* response to neutrophils (Figure 2A) because these cells produce larger respiratory bursts and greater levels of ROS than macrophages (Colombo et al., 2017; Forman and Torres, 2002; Thekkan et al., 2019). *P. aeruginosa* were incubated in conditioned medium from human neutrophils that were stimulated using phorbol 12-myristate 13-acetate (PMA), which triggers respiratory bursts (Karlsson et al., 2000). The expression of *fro* was quantified by averaging the ratios of YFP to mCherry fluorescence for at least one hundred individual *P. aeruginosa* cells. In support of our hypothesis, conditioned medium from PMA-stimulated neutrophils increased *fro* expression in bacteria by 30-fold compared to conditioned medium from unstimulated neutrophils (Figure 2B-C, Figure 2—source data 2). We confirmed that this *fro* response was not due to PMA alone, as PMA-treated medium without neutrophils had no effect on *fro* expression (Figure 2B-C). Similarly, conditioned medium from unstimulated neutrophils had no significant effect on *fro* expression compared to medium alone, suggesting that the upregulation of *fro* is due to respiratory burst products.

HOCl and other RCS are produced from ROS during respiratory bursts via myeloperoxidase (MPO). To investigate whether *P. aeruginosa fro* expression is induced by MPO-derived RCS, we pre-treated neutrophils prior to PMA stimulation with 4-aminobenzoic acid hydrazide (4-ABAH) (Figure 2D), a specific and irreversible MPO inhibitor (Kettle et al., 1997). Indeed, the activation of *fro* expression by PMA-stimulated neutrophil medium was abrogated in neutrophils that were pre-treated with the MPO inhibitor. The degree of *fro* inhibition by 4-ABAH followed a dose-dependent trend (Figure 2—figure supplement 1), consistent with a graded *fro* response to different RCS concentrations. HOCl production in neutrophils due to PMA stimulation and its inhibition by 4-ABAH was confirmed using a fluorometric HOCl-specific assay (Figure 2—figure supplement 2). These results indicate that *fro* upregulation depends on MPO activity and suggest that *fro* is activated specifically in response to HOCl.

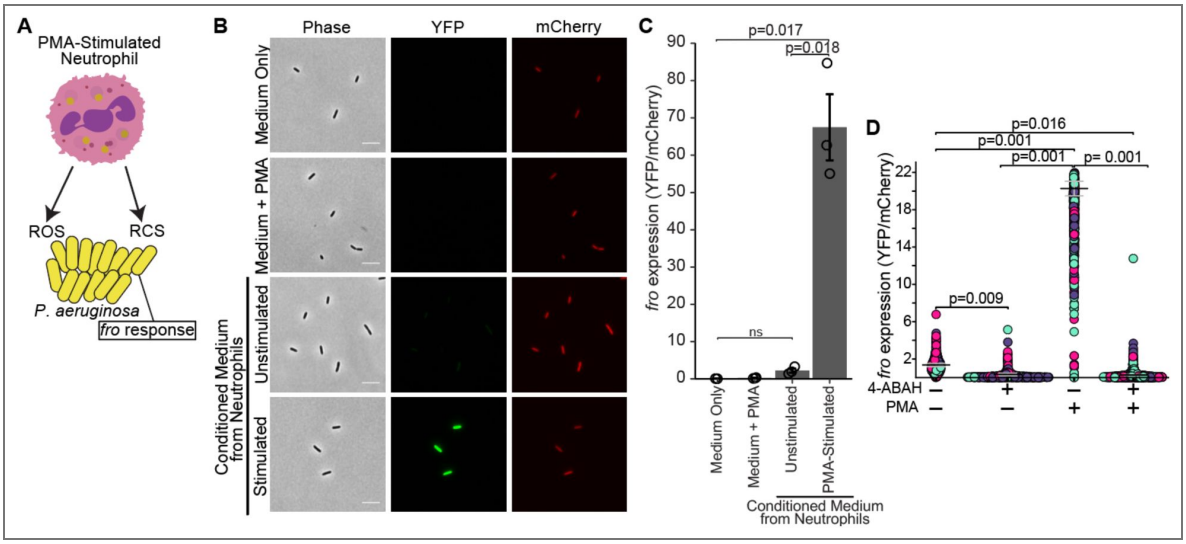


Figure 2. Conditioned medium from stimulated neutrophils induces *fro* expression.

(A) Schematic depicting that PMA-stimulated neutrophils produce respiratory burst products that could activate *fro* expression. **(B)** Representative phase-contrast and fluorescence images and **(C)** *fro* expression as determined by ratios of YFP to mCherry fluorescence, of *P. aeruginosa* strain AL143 cultured for 3 hours in medium only, medium with PMA, conditioned medium from PMA-stimulated neutrophils, or conditioned medium from unstimulated neutrophils. Unstimulated neutrophils have a value greater than 1. Scale bars represent 5 μm. Data points indicate an average from at least one hundred individual *P. aeruginosa* and gray columns indicate the mean of at least three independent experiments. **(D)** *fro* expression as determined by ratios of YFP to mCherry fluorescence of *P. aeruginosa* strain AL143 that was cultured for 3 hours in conditioned medium in which neutrophils were stimulated with 100 ng/mL PMA, pre-treated with 2mM 4-ABAH, or both. Data points indicate individual *P. aeruginosa* cells, with each color representing an independent experiment, bars represent the average of 3 independent experiments, each using separate neutrophil isolations, and error bars indicate standard error of the mean (SEM). P-values were obtained using Welch's t-test, with values of p>0.05 denoted as ns.

HOCl but neither H_2O_2 nor HNO_3 induces *fro* expression

ROS and RCS have inhibitory effects on bacterial growth. To explore the hypothesis that these classes of molecules induce *fro* expression in *P. aeruginosa*, we measured the effects of H_2O_2, HNO_3, and NaOCl on *fro* expression at concentrations approaching their minimum inhibitory concentrations (MICs) (Figure 3A). NaOCl increased *fro* expression 74-fold at 1 µM relative to untreated (Figure 3B, Figure 3B–Source Data). To validate that the increase in YFP intensity is due to increased transcription, reverse transcription quantitative PCR (RT-qPCR) was also performed, which yielded a 200-fold increase in *fro* transcription in response to 1 µM NaOCl treatment (Figure 3C). While 2 µM NaOCl caused only a 3-fold increase in *fro* expression, we attribute this to the effects of NaOCl on transcription and cell viability, as this concentration also decreased the constitutive mCherry reporter intensity and cell size (Figure 3—figure supplement 1).

To rule out that the individual sodium or chloride ion components of NaOCl were responsible for the increase in *fro* expression, we repeated experiments using NaCl, which yielded no change in *fro* expression (Figure 3D). To assess whether the *fro* response may be affected by pH, we tested NaOH at the same concentration as NaOCl, and at a concentration at which a change in pH could be discerned. Due to the robust buffering capacity of the medium, a pH change of ~0.1 required 6 mM NaOH, which is a concentration that had no obvious effect on growth but is significantly higher than the concentration of NaOCl needed to activate *fro* expression. Regardless, neither concentration of NaOH had any significant impact on *fro* expression (Figure 3D). As *fro* activation is not due to sodium or chloride ions nor changes in pH, we attributed the effects to hypochlorite (OCl^-), which is in equilibrium with hypochlorous acid (HOCl) at the pH of 7.1 in the medium used in our experiments.

To address the possibility that *fro* expression is activated by oxidation alone, we quantified *fro* expression in response to H_2O_2 and HNO_3, at concentrations up to their respective MICs (Figure 3A). While both are strong oxidizers, HNO_3 is not produced by neutrophils. Neither H_2O_2 nor HNO_3 induced any significant changes in *fro* expression up to near-MIC concentrations (Figure 3E). The lack of *fro* expression due to H_2O_2 treatment is not due to defects in fluorescent protein production, as H_2O_2 did not induce any change in *fro* transcript abundance, as assayed using RT-qPCR (Figure 3C).

Together, these indicate that strong oxidation alone is not sufficient to upregulate *fro*. We assessed whether other antimicrobial components of neutrophils or other bacterial stressors could be responsible for the *fro* response. Stimulated neutrophils release the antimicrobial peptide LL-37 and histones, which together constitute a synergistic antimicrobial attack (Doolin et al., 2020; Duong et al., 2025). Tobramycin is an aminoglycoside, a class of antibiotics that induces oxidative stress within bacteria (Kohanski et al., 2008). Neither the combination of LL-37 and histones nor tobramycin at near-MIC concentrations induced *fro* expression (Figure 3F). Together, these results indicate that *fro* expression is activated specifically by the neutrophil effector HOCl and not by intracellular oxidative stress or other neutrophil defense factors including LL-37, histones, or H_2O_2.

Corneal infection activates *fro* expression in bacteria

P. aeruginosa is a major cause of corneal ulcers and keratitis, which can cause visual impairment and blindness. Neutrophils are recruited within hours to corneal epithelial abrasions, with neutrophil cell density peaking at 18 hours (Li et al., 2006). We tested the hypothesis that *fro* expression is upregulated during corneal infection using a murine bacterial keratitis model in which *P. aeruginosa* strain PAO1F is inoculated onto a corneal abrasion, which we previously demonstrated results in significant neutrophil recruitment to the cornea (Minns et al., 2023; Ratitong et al., 2022). Mice corneas were harvested 2 or 20 hours post-infection and *fro* transcripts were quantified using RT-qPCR. Transcript abundance was normalized to the number of *P. aeruginosa* cells using 5S ribosomal RNA. In support of the hypothesis, *fro* expression increased significantly by 20 hours post-infection (Figure 4A). Expression levels showed no

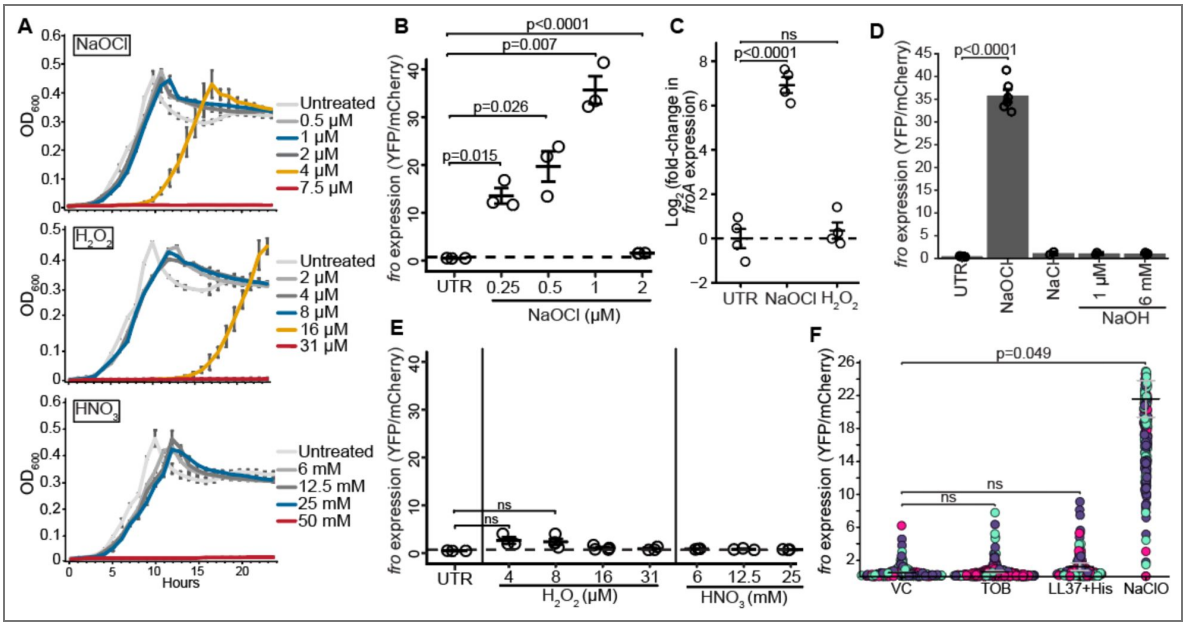


Figure 3. **NaOCl but neither H_2O_2 nor HNO_3 induces *fro* expression.**

(A) Growth profiles as measured by optical density (OD_{600}) of *P. aeruginosa* treated with the indicated concentrations of 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* after 3 hour treatment with the indicated concentrations of NaOCl or no treatment (UTR). The value for UTR is less than 1. **(C)** Abundance of *fro*A transcripts in *P. aeruginosa* after 30 minutes of 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 and E)** *fro* expression as determined by ratios of YFP to mCherry fluorescence in *P. aeruginosa* after 3 hour treatment with (D) 1 µM NaOCl, 1 µM NaCl, 1 µM NaOH, 1 µM or 6 mM NaOH, or no treatment, and **(E)** indicated concentrations of H_2O_2, HNO_3, or no treatment (data replotted from panel B). In panels B-E, data points indicate the average from at least one hundred individual *P. aeruginosa* cells, horizontal bars indicate the mean of independent experiments and error bars indicate SEM. Dashed lines indicate the average of the untreated condition, which was normalized to one. **(F)** *fro* expression as determined by ratios of YFP to mCherry fluorescence of *P. aeruginosa* after 3 hour treatment with 0.5 µg/mL tobramycin, vehicle control (VC; sterile water), 40 µg/mL, 1 µM NaOCl, or LL-37 and 10 µg/mL histones (LL37+His). Data points indicate individual *P. aeruginosa* cells, with different colors representing independent experiments. Horizontal bars indicate the mean of independent experiments and error bars indicate SEM. P-values were obtained using Welch's t-test, with values of p>0.05 denoted as ns. All data were acquired using the *P. aeruginosa* strain AL143.

significant change within the first two hours following infection, which we attribute to a lower level of immune cell recruitment during this period. The increase in *fro* expression 20 hours post-infection was not due to differences between the *P. aeruginosa* strains, because *fro* transcription in PAO1F also increased in response to NaOCl but not H_2O_2 (Figure 4—figure supplement 1), consistent with previous results.

Methionine sulfoxide reductase upregulation requires FroR

FroR is the σ factor required for activation of the *fro* operon in response to flow (Sanfilippo et al., 2019). To understand the response to HOCl, we performed transcriptional profiling of wild-type (WT) and Δ*froR P. aeruginosa* strains. The genes that were most upregulated by NaOCl in the WT strain compared to the Δ*froR* mutant were those in the *fro* operon, the phosphate and pyrophosphate-specific outer membrane porin genes *oprO* and *oprP,* and the methionine sulfoxide reductase genes *msrB, msrP,* and *msrQ* (Figure 5A and Appendix 1—table 1). Methionine sulfoxide reductases (MSRs) have a critical role in bacterial survival and defense against RCS stress. HOCl oxidizes methionine and cysteine residues 100-fold more rapidly than other amino acids (Pattison and Davies, 2006; Winterbourn, 1985) and oxidation of methionine in bacterial membrane proteins by neutrophil-produced HOCl correlates with increased bactericidal killing of *E. coli* and *S. aureus* (Rosen et al., 2009). HOCl oxidation of methionine forms methionine sulfoxide, which impairs protein function.

MSRs restore proper protein function by reducing methionine sulfoxide back to methionine (Chandra et al., 2023; Gray et al., 2013). Our data thus suggest that FroR is required for the upregulation of MSRs in response to HOCl stress. We did not observe upregulation in PA14_27570, which is homologous to MsrC, which reduces strictly free methionine sulfoxide, or in PA14_66330 (MsrA), which reduces both free and protein-bound methionine sulfoxide (Appendix 1—table 1). No significant changes in the expression of enzymes that repair cysteine oxidation, such as thioredoxin, glutathione redoxin, or disulfide repair, were observed, suggesting that the *fro* response is directed towards repairing methionine oxidation.

We reasoned that HOCl could oxidize methionine residues within a *P. aeruginosa* sensor, which could in-turn upregulate *fro* expression. A number of anti-σ factors contain sensory domains that transduce sensory signals to their cytoplasmic σ factor (Paget, 2015). FroI is an anti-σ factor for the FroR σ factor (Boechat et al., 2013), which raises the possibility that HOCl or its secondary products could oxidize FroI directly, resulting in the activation of FroR. Consistent with this possibility, we found that FroI contains the highest concentration of methionine and cysteine residues of all identified *P. aeruginosa* PA14 anti-σ factors (Appendix 2—table 1).

We hypothesized that *fro* activation would be suppressed in the presence of excess of free methionine, as it would reduce available HOCl and thus oxidation of the FroI sensor. Indeed, co-treatment of NaOCl with methionine suppressed *fro* expression (Figure 5B). We reasoned that other antioxidants such as cysteine and β-mercaptoethanol (βME) could also function as alternative oxidation targets for HOCl, and therefore also suppress *fro*. Consistent with this hypothesis, co-treatment of NaOCl with cysteine or βME significantly suppressed *fro* expression compared to cysteine or βME alone (Figure 5B and Figure 5—figure supplement 1A-B).

We considered how *fro* could be upregulated by stimulated neutrophils in the context of these findings. HOCl and other reactive chlorine species produced by neutrophils could oxidize the *P. aeruginosa* sensor, resulting in *fro* upregulation. Under this interpretation, supplying excess methionine, which would reduce HOCl, should also inhibit activation by neutrophils. Indeed, supplementing conditioned media from PMA-stimulated neutrophils with methionine completely suppressed *fro* expression (Figure 5C). Together, these data suggest a model in which *fro* responds to HOCl-induced oxidation of a sensor by upregulating MSRs.

FroR protects *P. aeruginosa* against HOCl

The upregulation of MSRs is expected to improve bacterial growth against HOCl stress. We measured the growth profiles of WT and Δ*froR* strains of *P. aeruginosa*. Treatment with 4 μM NaOCl significantly inhibited the growth of the wild-type strain (Figure 6A) but resulted in even

Figure 4. The expression of fro is activated during corneal infection

Abundance of *fro*A transcripts in *P. aeruginosa* strain PAO1F as measured by RT-qPCR, at 0, 2, or 20 hours post-inoculation onto abraded mouse corneas. 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 (time=0 hrs). Log_2(FC) indicates the log_2 of the fold-change. Data points represent independent corneal infections. Horizontal bars indicate the average of at least six independent experiments and error bars represent SEM. P-values were obtained using Welch's t-test, with values of p>0.05 denoted as ns.

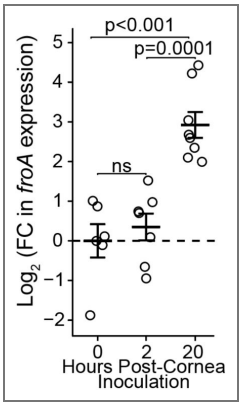
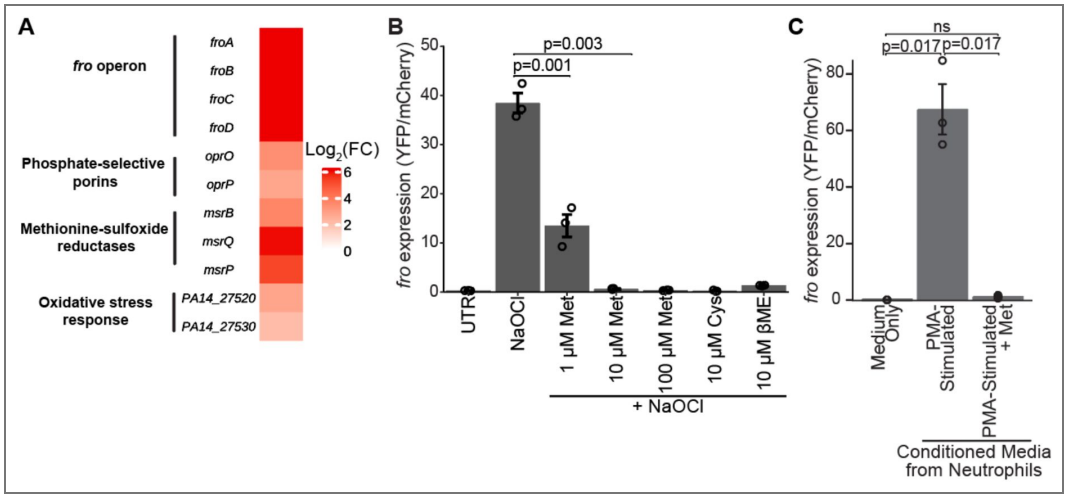


Figure 5. Expression of *fro* is inhibited by methionine and antioxidants.

(A) Heatmap showing *P. aeruginosa* gene transcripts (sorted by genomic locus) that were upregulated by at least 4-fold in the wild type PA14 background (strain AL143) compared to the Δ*froR* mutant (AL325) after 30 minutes of treatment with 1 μM NaOCl and that had p-values less than 0.05 (n≥3). Log_2(FC) indicates the log_2 of the fold-change. Raw data can be found online (Foik, Ilona P. et al., 2025) in NCBI's Gene Expression Omnibus (see Key Resources Table). **(B and C)** *fro* expression as determined by ratios of YFP to mCherry fluorescence in strain AL143 after 3 hour **(B)** treatment with; vehicle control (UTR), 1 μM NaOCl, or 1 μM NaOCl with combined with methionine (Met), cysteine (Cys), or β-mercaptoethanol (βME) at the indicated concentrations, and **(C)** incubation in medium only (replotted from Figure 2C), or conditioned medium from stimulated neutrophils with and without 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 Welch's t-test, with values of p>0.05 denoted as ns.



greater growth inhibition in the Δ*froR* strain. This observation suggests that the *fro* operon improves *P. aeruginosa* tolerance to HOCl stress.

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*) (Figure 6B and Appendix 1—table 2). 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 energy source, as evidenced by a concomitant upregulation of amino acid catabolism genes (*hpd*, *bkdA1*, *bkdA2*, and *bkdB*) by HOCl (Figure 6B and Appendix 1—table 2).

While the Δ*froR* mutant was more sensitive to HOCl than WT, its growth was not fully inhibited (Figure 6A), suggesting that additional mechanisms relieve HOCl stress. In the Δ*froR* mutant, HOCl upregulated genes encoding multidrug efflux pumps (*mexEF-oprN* and *mexXY*) (Figure 6C and Appendix 1—table 3). These pumps can relieve oxidative stress (Fargier et al., 2012; Fraud and Poole, 2011) but were not activated in the WT strain. This observation suggests that bacterial defense against HOCl may be hierarchical, in which Fro-regulated and Mex pathways could be first-line and second-line responses to HOCl stress, respectively. Supporting this interpretation, it has been reported that treatment of the WT strain using significantly higher (millimolar) HOCl concentrations upregulates *mexEF-oprN* (Farrant et al., 2020) and the *mexXY* regulator *mexT* (Groitl et al., 2017).

Discussion

Neutrophils are activated by the presence of bacteria that infect host tissue. In their activated state, neutrophils generate respiratory bursts that produce an abundance of HOCl. We have demonstrated that *P. aeruginosa* responds to activated neutrophils by upregulating the transcriptional expression of the *fro* operon, which relieves HOCl stress. Our data suggest that the upregulation of *fro* could function as a bacterial defense mechanism against neutrophil attack, thus improving *P. aeruginosa* survival and colonization of human tissue during infection.

Detection and response to HOCl

Based on (i) our observation that the expression of multi-drug efflux pumps is activated in the absence of FroR (Figure 6C and Appendix 1—table 3), (ii) that this expression occurs at high HOCl concentrations (Farrant et al., 2020), and (iii) that multiple systems repair HOCl damage, the HOCl response could follow a hierarchy of activation. Our data suggest a model in which the *fro* system is the first-line response to HOCl. FroR activates two distinct classes of reductases that repair HOCl-oxidized methionine in proteins: MsrB reduces methionine sulfoxide in the cytoplasm using electrons provided by the thioredoxin system (Ezraty et al., 2005; Lourenço Dos Santos et al., 2018), while MsrPQ works in the periplasm and inner membrane using electrons from the respiratory chain (Gennaris et al., 2015). The activation of these two reductase types enables *P. aeruginosa* to limit HOCl stress in separate cellular spaces by utilizing distinct electron donor sources.

How does *P. aeruginosa* detect HOCl? As an anti-σ factor, FroI could immobilize FroR, inhibiting FroR from reaching transcriptional targets. Given the particularly high density of methionine and cysteine residues in the FroI, this protein could serve as an HOCl sensor by releasing the sigma factor FroR in response to oxidation of these residues (Figure 6D). While our data shows that HOCl can directly activate *fro* expression, HOCl reacts with neutrophil components, forming secondary RCS, including monochloramines, protein-derived chloramines, and taurine chloramine (Kim and Cha, 2014; Klebanoff et al., 2013; Ulfig and Leichert, 2020). Taurine chloramine is an expected major RCS released from neutrophils due to the high abundance of taurine in these cells (Kim and Cha, 2014; Marcinkiewicz and Kontny, 2014). Because it retains oxidizing

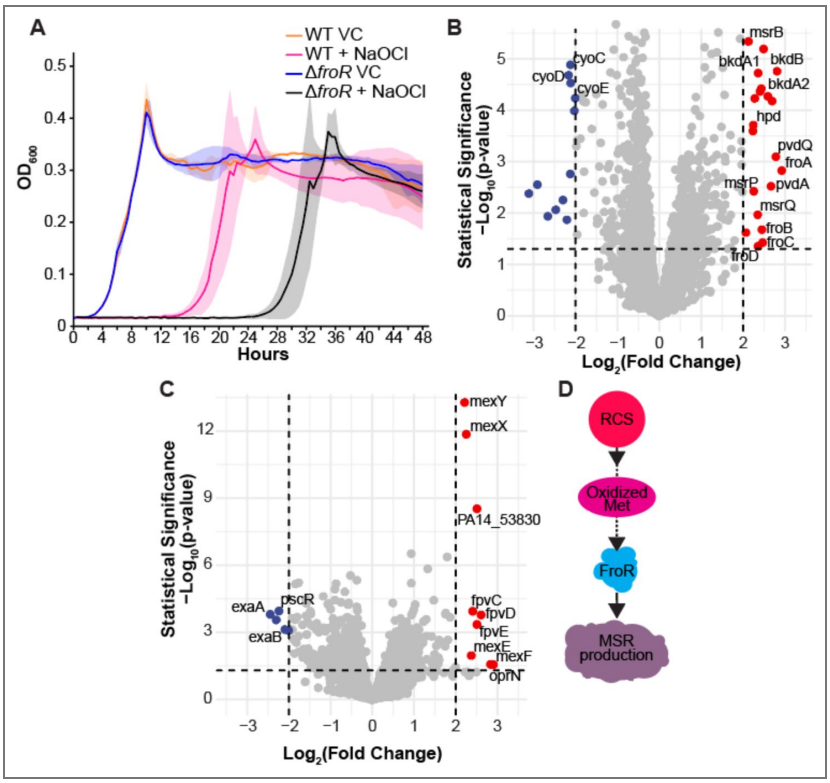


Figure 6. FroR improves P. aeruginosa growth against HOCl stress and regulates transcription of genes involved in antioxidant defense.

(A) Growth profiles of wild-type (WT) *P. aeruginosa* strain AL143 and the Δ*froR* mutant strain AL325 treated with 4 μM NaOCl or vehicle control (VC). 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)** Δ*froR* after treatment with 1 μM NaOCl (n≥3), as determined by RNA sequencing. Genes in red (upregulated) and blue (downregulated) were altered by at least four-fold. The unlabeled gene loci shown have not been previously annotated. P-values were determined using the Wald test (Love et al., 2014). Greater values along the vertical axis indicate greater statistical significance. P-values were determined using the Wald test (Love et al., 2014). Greater values along the vertical axis indicate greater statistical significance and the dashed horizontal line indicates a p-value of 0.05. Raw data can be found online (Foik, Ilona P. et al., 2025) in NCBI's Gene Expression Omnibus (see Key Resources Table). **(D)** Schematic that depicts a model in which RCS oxidizes methionine residues (Met) in a target sensor(s), such as the ECF anti-σ factor FroI, which in turn activates the FroR σ factor and increases the production of methionine sulfoxide reductases (MSR).

potential towards cysteine and methionine (Peskin and Winterbourn, 2001), taurine chloramine could contribute to the activation of *fro* expression alongside HOCl. Future work will need to assess the relative efficacies of HOCl and other secondary RCS towards activating *fro* expression.

Relationship with neutrophil-mediated killing and flow

One of the striking features of the Fro system is that it is tuned for maximal activation at micromolar HOCl concentrations (1 μM) that are close to the MIC. While our experiments measured the effects of HOCl at steady-state using medium that does not contain amino acids, host proteins, and other host factors, HOCl dynamics in host environments are much more complex, where HOCl production is estimated to be in quantities that exceed the sensitivity of Fro. For example, HOCl production is predicted in excess of 100 mM per minute in the phagosome (Winterbourn et al., 2006). In addition, stimulated neutrophils at densities comparable to those found in blood (Tahir and Zahra, 2026) using PMA produces extracellular HOCl at 80 μM per hour, as measured through taurine chloramine absorbance (Weiss et al., 1982). Additional measurements using slight changes in stimulation time, neutrophil density, or PMA concentration yielded comparable HOCl production rates within a factor of 2 (Dypbukt et al., 2005; Test et al., 1984). At sites of inflammation, HOCl production in interstitial fluid has been predicted to be in the millimolar range due to the greater density of neutrophils there (Weiss, 1989).

Despite the high production rates of HOCl, the steady-state concentration in neutrophils and *in vivo* is expected to be significantly lower due to its rapid conversion into secondary RCS through reaction with neutrophil or plasma components. For example, the lifetime of HOCl is estimated to be 0.1 μs and have an action radius under 0.1 μm in the phagosome (Schürmann et al., 2017), which would mitigate the antimicrobial effects of HOCl but retain oxidizing potential. The secondary RCS taurine chloramine is long-lived, well-tolerated in the body, and retains significant bactericidal activity in the micromolar range (Gottardi and Nagl, 2010; Kim and Cha, 2014; Nagl et al., 2000). It is plausible that diminished HOCl or longer-lived secondary RCS such as taurine chloramine fall within the activating range of Fro, which would in turn mitigate their antimicrobial effects through upregulation of the MSRs. Importantly, our observation that *fro* expression is activated during corneal infection, which involves significant neutrophil recruitment, supports a role for the Fro response in a neutrophil-dense environment.

An additional layer of complexity of understanding the role of Fro during infection is that flow exposes bacteria to higher concentrations of molecules in a fluid. The bacterial response to flow, which was initially referred to as rheosensing (Sanfilippo et al., 2019), was more recently attributed to the effects of molecular transport by flow (Padron et al., 2023). In particular, flow stimulated a response to H_2O_2 in cells that were otherwise unresponsive to it in the absence of flow (Padron et al., 2023). Flow could similarly amplify the stimulating effects of RCS, causing *P. aeruginosa* to respond to circulating neutrophils that produce RCS at much lower concentrations. At the same time, flow could amplify the bactericidal effects against *P. aeruginosa*. Future work will need to determine the relative steady-state abundances of different secondary RCS and address their impact in combination with flow on bactericidal activity and the activation of the Fro response.

At the fundamental level, Fro is a system that increases antimicrobial tolerance, which can promote antimicrobial resistance. For example, bacteria that are exposed to low concentrations of HOCl have higher rates of horizontal gene transfer and can acquire antibiotic resistance genes more rapidly (Hou et al., 2019; Jin et al., 2020). The Fro system could thus facilitate antibiotic resistance through increasing *P. aeruginosa* tolerance to HOCl. Our findings suggest that the bacterial HOCl response could thus be an important bacterial defense mechanism to target in the development of novel antibiotic therapeutics. Inhibition of this pathway could increase *P. aeruginosa* susceptibility to neutrophil-mediated killing while minimizing antibiotic resistance.

Materials and methods

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
strain, strain background (Pseudomonas aeruginosa)	AL143, UCBPP-PA14	Gitai lab (Sanfilippo et al., 2019)		PA14 P_{fro}-*yfp attB*::[$P_{A1/04/03}$-*mCherry*]
strain, strain background (Pseudomonas aeruginosa)	AL325, UCBPP-PA14	Gitai lab (Sanfilippo et al., 2019)	Δ*froR*	PA14 P_{fro}-*yfp attB*::[$P_{A1/04/03}$-*mCherry*] Δ*froR*
strain, strain background (Pseudomonas aeruginosa)	PAO1F	Pearlman lab and (Vance et al., 2005; Vareechon et al., 2017)		
strain, strain background (Mus musculus)	C57BL/6J; strain #000664	Jackson Laboratory, Bar Harbor, ME	RRID: IMSR_JAX:000664	mice aged 6-8 weeks
biological sample (Homo sapiens, M&F)	Primary Human neutrophils	Obtained through the Institute for Clinical and Translational Science blood donor program at the University of California, Irvine through approved protocols with the UCI Institutional Review Board HS#2001-2058. Informed consent was obtained from all donors.		Freshly isolated from whole blood samples obtained from an equal ratio of male and female donors aged 18-65.
cell line (Mus musculus, F)	J774A.1	American Type Culture Collection, Manassas, VA	ATCC TIB-67	
antibody	PE-CD66b (mouse monoclonal)	Fisher Scientific, Waltham, MA	Invitrogen CD66b Monoclonal Antibody (G10F5), PE-Cyanine7, eBioscience #25066641	
antibody	FITC-CD16 (mouse monoclonal)	Fisher Scientific, Waltham, MA	Invitrogen CD16 Monoclonal Antibody (eBioCB16 (CB16)), FITC, eBioscience #509462	
antibody	APC-CD11b (mouse monoclonal)	Fisher Scientific, Waltham, MA	Invitrogen™ CD11b Monoclonal Antibody (ICRF44), APC-eFluor™ 780, eBioscience™, Invitrogen™ #5016145	

sequence-based reagent	PA14_froA_v2_F	This study	5'-TTTCCCTCGCTTC**C**TCCGTC-3'	Targets PA14_21570 in PA14 and PA3284 in PAO1. Bold indicates a mismatch with the PAO1 target.
sequence-based reagent	PA14_froA_v2_R	This study	5'-ACCTTCCTTGGC**C**TTCTCGG-3'	Targets PA14_21570 in PA14 and PA3284 in PAO1. Bold indicates a mismatch with the PAO1 target.
sequence-based reagent	PAO1_PA14_5S_F	This study	5'-TAGAGCGTTGGAACCACCTG-3'	Targets 5S rRNA in both PAO1 and PA14
sequence-based reagent	PAO1_PA14_5S_R	This study	5'-GAGACCCCACACTACCATCG-3'	Targets 5S rRNA in both PAO1 and PA14
peptide, recombinant protein	histones	Sigma-Aldrich, St. Louis, MO	Calf thymus whole histone type II-A #H9250-100MG	
commercial assay or kit	NucleoSpin RNA kit	Fisher Scientific, Waltham, MA	Macherey-Nagel NucleoSpin RNA #NC9581114	
commercial assay or kit	Fluorometric HOCl detection assay	AAT Bioquest, Pleasanton, CA	Amplite Fluorometric Hypochlorite Assay Kit #13846	
commercial assay or kit	NEBNext Multiplex Oligos for Illumina (Index Primers Set 1)	New England Biolabs, Ipswich, MA	#E7335S	
commercial assay or kit	NEBNext Multiplex Oligos for Illumina (Index Primers Set 2)	New England Biolabs, Ipswich, MA	#E7500S	
commercial assay or kit	NEBNext rRNA Depletion Kit (Bacteria) with RNA Sample Purification Beads	New England Biolabs, Ipswich, MA	#E7860L	
commercial assay or kit	NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads	New England Biolabs, Ipswich, MA	#E7765S	
commercial assay or kit	High-Capacity cDNA Reverse Transcriptase Kit	Fisher Scientific, Waltham, MA	Applied Biosystems #4368814	

chemical compound, drug	HNO_3	Sigma-Aldrich, St. Louis, MO	Nitric acid ACS reagent, 70% #438073	
chemical compound, drug	NaOH	Fisher Scientific, Waltham, MA	Sodium Hydroxide Pellets #S3181	
chemical compound, drug	cysteine	Sigma-Aldrich, St. Louis, MO	L-cysteine #C7352	
chemical compound, drug	β-mercaptoethanol	Fisher Chemicals, Waltham, MA	#O3446I100	
chemical compound, drug	NaCl	Sigma-Aldrich, St. Louis, MO	#S9888	
chemical compound, drug	methionine	Sigma-Aldrich, St. Louis, MO	#M5308	
chemical compound, drug	H_2O_2	Fisher Chemicals, Waltham, MA	Hydrogen Peroxide, 3% #H324-500	
chemical compound, drug	NaOCl	Fisher Scientific, Waltham, MA	Sodium hypochlorite 4.5 to 5.5% Reagent Grade #AC419550250	
chemical compound, drug	NaOCl	Sigma-Aldrich, St. Louis, MO	Sodium Hypochlorite Solution reagent grade, available chlorine 4.00-4.99 % #239305	
chemical compound, drug	NaOCl pentahydrate	Tokyo Chemical Industry America	Sodium Hypochlorite Pentahydrate #S0939	
chemical compound, drug	Tobramycin	Fisher Scientific, Waltham, MA	Tobramycin, 97%, Acros Organics #AC455430010	
chemical compound, drug	Dextran	Sigma-Aldrich, St. Louis, MO	dextran (from Leuconostoc spp, Mr 450,000-650,000) #501780721	
chemical compound, drug	Ficoll	Fisher Scientific, Waltham, MA	Cytiva Ficoll-Paque™ PLUS Media #45001749	
chemical compound, drug	Ficoll	VWR, Radnor, PA	Ficoll-Paque reg PLUS and Ficoll-Paque reg PREMIUM Centrifugation Media, GE Healthcare #95021-205	
chemical compound, drug	RBC Lysis Buffer	Fisher Scientific, Waltham, MA	Invitrogen™ eBioscience™ 1X RBC Lysis Buffer #00433357	
chemical compound, drug	DMEM	Fisher Scientific, Waltham, MA	Gibco™ DMEM, high glucose, pyruvate #11995065	
chemical compound, drug	PBS	Fisher Scientific, Waltham, MA	Gibco PBS #10010072	

chemical compound, drug	RPMI 1640	ATCC, Manassas, VA	#50188266NP	
chemical compound, drug	DPBS	Fisher Scientific, Waltham, MA	Gibco DPBS, no calcium, no magnesium #14190144	
chemical compound, drug	14mL polystyrene round-bottom tubes	Corning, Corning, NY	Falcon 14 mL Round Bottom Polystyrene Test Tube #352051	
chemical compound, drug	black polystyrene 96-well clear-bottom microplates	Fisher Scientific, Waltham, MA	Corning 96-Well, Cell Culture-Treated, Flat-Bottom Microplate #7200588	
chemical compound, drug	SsoAdvanced Universal SYBR Green Supermix	BioRad, Hercules, CA	#725271	
chemical compound, drug	Propidium Iodide	Sigma-Aldrich, St. Louis, MO	Propidium iodide solution #P4864	
chemical compound, drug	Casamino acids	Fisher Scientific, Waltham, MA	Gibco Bacto Casamino Acids #DF0230173	
chemical compound, drug	Glucose	Fisher Chemicals, Waltham, MA	Dextrose (D-Glucose), Anhydrous #D16500	
chemical compound, drug	Citrate	Fisher Chemicals, Waltham, MA	#S279500	
chemical compound, drug	K_2HPO_4	Fisher Chemicals, Waltham, MA	Potassium Phosphate Dibasic Anhydrous # P288500	
chemical compound, drug	KH_2PO4	Fisher Chemicals, Waltham, MA	Potassium Phosphate Monobasic #P285500	
chemical compound, drug	$(NH_4)_2SO_4$	Fisher Chemicals, Waltham, MA	Ammonium Sulfate #A702500	
chemical compound, drug	$MgSO_4$	Sigma-Aldrich, St. Louis, MO	Magnesium sulfate heptahydrate, ACS reagent #230391	
chemical compound, drug	PMA	Sigma-Aldrich, St. Louis, MO	Phorbol 12-myristate 13-acetate #P1585	
chemical compound, drug	4-ABAH	Sigma-Aldrich, St. Louis, MO	4-Aminobenzoic hydrazide #A4190910G	
chemical compound, drug	$NaH_2PO_4 \cdot H_2O$	Sigma-Aldrich, St. Louis, MO	Sodium phosphate monobasic monohydrate #S9638	
chemical compound, drug	Taurine	Sigma-Aldrich, St. Louis, MO	#T8691	
chemical compound, drug	Cacl2	Sigma-Aldrich, St. Louis, MO	calcium chloride anhydrous #C1016	

chemical compound, drug	MgCl2	Sigma-Aldrich, St. Louis, MO	magnesium chloride anhydrous # M8266	
chemical compound, drug	KCl	Sigma-Aldrich, St. Louis, MO	potassium chloride, anhydrous #746436	
chemical compound, drug	NaCl	Sigma-Aldrich, St. Louis, MO	Sodium chloride # S9888	
chemical compound, drug	Na2HPO4	Fisher Chemicals, Waltham, MA	dibasic sodium phosphate heptahydrate #S373500	
chemical compound, drug	Agar	BD Biosciences, Franklin Lakes, NJ	Bacto Agar	
chemical compound, drug	Tris base	Fisher Chemicals, Waltham, MA	2-Amino-2-(hydroxymethyl)-1,3-propanediol #BP152	
chemical compound, drug	agarose	Fisher Scientific, Waltham, MA	Invitrogen UltraPure Agarose #16500500	
chemical compound, drug	EDTA	Fisher Chemicals, Waltham, MA	Ethylenediamine Tetraacetic Acid, Disodium Salt Dihydrate, 99.0 to 101.0% #S311500	
chemical compound, drug	Lysozyme	Sigma-Aldrich, St. Louis, MO	Lysozyme from chicken egg white #L6876	
chemical compound, drug	SDS	Sigma-Aldrich, St. Louis, MO	Sodium dodecyl sulfate #L3771	
chemical compound, drug	LL-37	Peptide Sciences	#CAP-18	
software, algorithm	Custom scripts used in MATLAB	(asirya and YonghanWu, 2025) Matlab scripts on Github		
software, algorithm	MATLAB	Mathworks, Natick, MA	Version R2024b	
software, algorithm	fastQC	(Simon Andrews, n.d.) (Simon Andrews, n.d.)	Version 0.21.1	
software, algorithm	Trimmomatic	(Bolger et al., 2014) (Bolger et al., 2014)	version 0.39	
software, algorithm	Java	Oracle Corporation, Austin, Texas	V1.8.0	
software, algorithm	Bowtie2	(Langmead and Salzberg, 2012) (Langmead and Salzberg, 2012)	version 2.5.4	
software, algorithm	Subread	(Liao et al., 2013) (Liao et al., 2013)	version 2.0.8	
software, algorithm	DeSeq2	(Love et al., 2014) (Love et al., 2014)	version 1.40.2	

software, algorithm	GOEnrichment	Gene Ontology Consortium, 2021	version 2.0.1	
software, algorithm	R	The R Foundation, Vienna, Austria	Version 4	
software, algorithm	RStudio	Posit Software, Boston, MA	2026.01.2+418 "Apple Blossom" Release	
software, algorithm	Excel	Microsoft, Redmond, WA		
other	RNA-seq raw data	(Foik, Ilona P. et al., 2025) NCBI Gene Expression Omnibus	GEO Series accession number GSE290217	
other	Nikon Eclipse Ti-E microscope	Nikon, Melville, NY		
other	Sola light engine	Lumencor, Beaverton, OR		
other	Nanodrop 2000	Thermo Fisher, Waltham, MA		
other	BioTek Synergy HTX plate reader	BioTek, Winooski, VT		
other	Illumina NovaSeq X Plus	Used through the UC Irvine Genomics Research & Technology Hub Illumina, San Diego, CA		
other	Orca Flash 4.0 V2 camera	Hamamatsu, Bridgewater, NJ		
other	C1000 Thermal Cycler with a CFX96 real-time detection system	BioRad, Hercules, CA		

Bacterial Culture Conditions

Strains were struck out from frozen stocks maintained at -80°C onto plates containing LB-Miller broth and 2% Bacto agar and incubated overnight at 37°C. Single colonies were inoculated into 2mL of modified MinA minimal medium (60.3mM K_2HPO_4, 33.0mM KH_2PO4, 7.6mM $(NH_4)_2SO_4$, 1.0mM $MgSO_4$; (Malke, 1993) containing 0.2% (w/v) sodium citrate (hereafter referred to as MinA minimal medium), and cultured for 18 hours in a roller drum at 17 rpm and 37 °C.

Imaging *P. aeruginosa* under flow

Microfluidic devices were fabricated using standard soft lithography techniques as established previously (Siryaporn et al., 2015) with channel dimensions of 100 x 50 µm (width x height). *P. aeruginosa* was cultured for 18 hours in LB-miller medium, diluted 1:100, cultured to mid-exponential phase, and injected into the microfluidic device. Fresh LB was injected at a constant rate of 10µL/min. Images were acquired as described in the fluorescence microscopy section.

Neutrophil Isolation

Neutrophils were isolated freshly from whole blood as described in (Clark et al., 2018). Briefly, 3% dextran in PBS was used to separate red blood cells (RBCs) from whole blood. Neutrophils were purified from the remaining cells by overlaying on a Ficoll density gradient and centrifugation in for 30 minutes at 500 × g. RBCs were lysed using RBC Lysis Buffer and centrifugation for 5 mins at 300 × g. Supernatant was aspirated and the remaining neutrophil pellets were resuspended in RPMI 1640 medium prior to normalizing cell counts. Neutrophils were used immediately for stimulation, preparation of conditioned media, or co-incubation experiments with *P. aeruginosa* as described below, and purity was assessed in parallel using the fluorescent antibodies APC-CD11b, FITC-CD16 and PE-CD66b and a ACEA NovoCyte Flow Cytometer. This procedure routinely yielded greater than 95% CD11b-positive neutrophil populations.

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

Neutrophils were prepared as described above. J774A.1 mouse macrophages were cultured in 10 mL DMEM in T75 flasks at 37 °C with 5% CO_2. Macrophages were passaged every three days through scraping and were passaged every 5 days through trypsinization. Mammalian cells were washed three times by centrifugation at 300 × g for 5 minutes and resuspension in DPBS, and 1 mL aliquots of $4x10^5$ cells were transferred into flat-bottom dishes. *P. aeruginosa* overnight (~18h) cultures were centrifuged at 4600 × g for 2 minutes, resuspended in DPBS three times, diluted to a final OD_{600} of 0.2, added to the mammalian cells at a multiplicity of infection of 10, and incubated for 30 minutes at 37°C. Dishes were aspirated until approximately 20 µL of medium remained. 1% agarose pads containing DPBS were placed on top of the samples in the dishes to immobilize bacteria and cells. Samples were imaged as described in the fluorescence microscopy section.

Conditioned Medium from Neutrophils

To activate neutrophils, 2 million freshly harvested neutrophils per mL in 10mM phosphate buffer (pH 7.4; 2.46 mM monobasic phosphate, 7.54 mM dibasic sodium phosphate) containing 140 mM sodium chloride, 10 mM potassium chloride, 0.5 mM magnesium chloride, 1 mM calcium chloride, 1 mg/mL glucose, and 5 mM taurine (Dypbukt et al., 2005), were treated with 100 ng/mL of PMA in 14mL polystyrene round-bottom Falcon tubes for 1 hour at 37°C and 17 rpm. For MPO inhibition studies, neutrophils were pre-treated with 2 mM of 4-ABAH for 30 mins prior to addition of PMA. This concentration of 4-ABAH was scaled up based on its usage of 500 µM of 4-ABAH to treat one million human neutrophils per mL (Tseng et al., 2018). Supernatant was collected after centrifugation for 5 minutes at 4,600 × g and 24 °C and used as conditioned medium immediately for HOCl detection assay as described below. In parallel, *P. aeruginosa* that were cultured for 18 hours in MinA minimal medium were diluted 1:1000 into conditioned media and were incubated for 3 hours at 37 °C in a rolling drum at 17 rpm and imaged as described in the fluorescence microscopy section.

HOCl Fluorometric Detection Assay

Conditioned medium was assessed immediately using the Amplite Fluorometric Hypochlorite Assay Kit according to manufacturer protocol. Samples were incubated at room temperature in the dark for 10 minutes and measured for fluorescence using a BioTek Synergy HTX plate with 540/25 nm and 585/10 nm filters for excitation and emission, respectively, and using the autogain set to the 1 µM NaOCl condition. HOCl concentrations were determined by fitting the calibration values to the Hill equation using a three-parameter fit and the lowest value in the data series as the minimum value. HOCl concentrations were background-subtracted by the neutrophil buffer-only sample.

Treatments of *P. aeruginosa* with oxidizers, antioxidants, and stressors

P. aeruginosa were cultured 18 hours in MinA minimal medium and diluted 1:1000 into the same medium. HOCl, H_2O_2, HNO_3, NaOH, NaCl, methionine, L-cysteine, or β-mercaptoethanol (βME) were added to 50 mL cultures at the concentrations indicated in the main text and were incubated in 250 mL flasks for 3 hours at 37 °C in an orbital shaker at 225 rpm. LL-37, histones and tobramycin were added following the 1:1000 diluted and incubated in 14 mL falcon tubes in a roller drum at 17 rpm. Cultures were imaged and analyzed as described in the fluorescence microscopy section.

Fluorescence and Phase Contrast Microscopy

P. aeruginosa were immobilized on 1% agarose pads containing MinA minimal medium and imaged immediately. Cultures containing densities below 10 *P. aeruginosa* per frame were concentrated using a syringe filter and imaged immediately. Experiments in Figures 2D and

3F were performed without the syringe filter in order to confirm that the *P. aeruginosa* response was not affected by the filtering procedure.

Imaging

Images were acquired using Nikon NIS-Elements software and a Nikon Eclipse Ti-E microscope containing a Nikon 100X Plan Apo (1.45 N.A.) objective, Nikon Ph3 phase contrast condenser annulus, Sola light engine, Hamamatsu Orca Flash 4.0 V2 camera, 459/526/596 dichroic, 575/25 excitation and 641/75 emission filters for mCherry, and 509/22 excitation and 544/24 emission filters for YFP (Semrock, Rochester, NY).

Image Analysis

Images were analyzed in MATLAB using custom built software written previously (see the Key Resources Table to download). Briefly, *P. aeruginosa* masks were determined from phase contrast images using an edge-detection algorithm. For size analysis, the total pixel area of each individual *P. aeruginosa* cell was determined by computing the mask area and converting from pixels to μm^2 by multiplying the mask area by a factor of 0.004225 μm^2/pixel to account for the microscope camera pixel size and objective magnification. *Fro* expression was quantified by averaging computed ratios of YFP to mCherry fluorescence from the masked areas for at least one hundred individual *P. aeruginosa* cells per condition for each experiment.

Bacterial Growth Curves

Overnight cultures of bacteria were diluted into MinA minimal medium to an optical density at 600 nm (OD_{600}) of 0.01, given treatments, and incubated in sterile 96-well microplates at 37°C in a Synergy HTX multi-mode plate reader with continuous orbital shaking at 180 rpm and a 3mm amplitude, with OD_{600} readings taken every 30 minutes.

Reverse Transcription Quantitative PCR (RT-qPCR)

For non-murine model samples, bacterial overnight cultures were diluted 1:1000 into fresh MinA medium and incubated for 2.5 hours at 37°C and 225 rpm preceding a 30 minute treatment with 1 µM NaOCl or vehicle control. Samples were concentrated using syringe filters and centrifuged at 13,000 × g for 4 minutes. Pellets were snap-frozen in liquid nitrogen and stored at -80C before performing RNA extraction with the NucleoSpin RNA kit. cDNA was prepared from the using the High-Capacity cDNA Reverse Transcriptase Kit, and quantitative PCR was performed using the SsoAdvanced Universal SYBR Green Supermix using the C1000 Thermal Cycler with a CFX96 real-time detection system. *froA* transcripts were quantified using the primers PA14_froA_v2_F and PA14_froA_v2_R that target PA14_21570 in PA14 and PA3284 in PAO1 (see Key Resources Table). Transcript abundances for each sample were normalized by 5S rRNA abundance using the primers PAO1_PA14_5S_F and PAO1_PA14_5S_R (see Key Resource Table), yielding normalized count thresholds (Cts). The average fold change in transcript abundance was determined by computing the ratio of the averaged normalized Cts and raising by the power of 2, as performed previously (Bru et al., 2019). All primers were validated using a standard calibrating curve to assess Cts and transcript abundance.

Murine Model of Corneal Infection

Expression of *froA* was assessed in a murine model of corneal infection as described previously (Minns et al., 2023; Sun et al., 2012). The work received prior approval from the Institutional Animal Care and Use Committee at the University of California Irvine under protocol #AUP-21-123. Briefly, *P. aeruginosa* strain PAO1F was cultured to mid-exponential phase in MinA minimal medium that was modified to contain only 0.05% citrate and supplemented with 0.1% casamino acids and 0.2% glucose as the carbon sources, centrifuged, and resuspended in PBS. Corneal epithelia of C57BL/6J mice aged 6-8 weeks were abraded with 3x5 mm scratches using a 25G needle, and 2 µL of PBS containing $5x10^4$ of *P. aeruginosa* strain PAO1F was applied topically. Mice were selected at random and those displaying signs of illness or distress were excluded from the study. Comparable numbers of male and female mice were used in each experimental group. The

number of animals used for each experiment was determined based on a power analysis estimate. Mouse eyeballs were collected and homogenized in 1 mL of PBS 2 or 20 hours post-infection and centrifuged for 5 minutes at 150 × g to remove corneal materials. Supernatant was collected and centrifuged for 4 minutes at 13,000 × g. Pellets containing bacteria were suspended in lysis solution (10 mM Tris-HCl, 1 mM EDTA pH 8.0, 0.5 mg/mL lysozyme, 1% SDS) (Bru et al., 2019) RNA was prepared using the NucleoSpin RNA kit and assessed for mRNA expression using RT-qPCR. Pellets containing bacteria were suspended in lysis solution (10 mM Tris-HCl, 1 mM EDTA pH 8.0, 0.5 mg/mL lysozyme, 1% SDS) (Bru et al., 2019).

Samples were not blinded because they were processed as they were collected at different timepoints, which revealed their identity.

RNA-seq Library Preparation and Data Analysis

P. aeruginosa was cultured and RNA was prepared as described in the RT-qPCR section. RNA yield was measured using a Nanodrop 2000. Samples were depleted of ribosomal RNA using the NEBNext rRNA Depletion kit, from which cDNA libraries were constructed using the NEBNext Ultra II Directional Library kit, which were sequenced by the UC Irvine Genomics Research & Technology Hub (Irvine, CA) using an Illumina NovaSeq X Plus using paired-end 150 bp reads at a depth of approximately 10 million reads per sample. Raw reads were checked with fastQC and trimmed and filtered into paired and unpaired reads using Trimmomatic in Java using the 'PE' setting. Paired and unpaired reads were aligned to the PA14 genome (NCBI accession NC_008463.1) using Bowtie2 using the default 'sensitive' mode, were counted using the featureCounts program in Subread with the 'countReadPairs' option enabled for paired-end reads, and were fit into a negative binomial model to compute fold-change in gene expression using DeSeq2. The statistical significance of the changes were computed using the Wald test in DeSeq2. Gene Ontology enrichment analysis for Figure 1A was performed using GOEnrichment.

Statistical Analyses

Statistical analyses and figures were generated using Excel, R, and RStudio.

Data availability

Raw data for all figures that contain statistical analyses are available in the supplemental source data files and appendices. All other datasets and software are described in the Key Resources Table.

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Additional information

Author Contributions

IPF, SJK, RS and AS conceived and designed the experiments. IPF, SJK, RS, SA, MEM, LAU, LD, and APH performed experiments and analyzed data. IPF and LAU wrote the initial draft of the manuscript. SJK and AS revised it. All authors edited the paper.

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EC | Horizon Europe | Excellent Science | HORIZON EUROPE Marie Sklodowska-Curie Actions (MSCA)	https://doi.org/10.3030/847413	Ilona P Foik
Ministry of Science and Higher Education	5005/H2020-MSCA-COFUND/2019/2	Ilona P Foik
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Additional files

Supplementary Materials

Source Data

References

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Peer reviews

Reviewer #1 (Public review):

Summary:

Foik et al. report that hypochlorous acid, a reactive chlorine species generated during host defense, activates the transcription of the froABCD in P. aeruginosa. This gene cluster had previously been associated with a potential role during flow of fluids and appears to be regulated by the sigma factor FroR and its anti-sigma factor FroI. In the present study, the authors show that froABCD is expressed both in neutrophils and macrophages, which they claim is likely a result of HOCl but not H2O2 production. Fro expression is also induced in a murine model of corneal infection, which is characterized by immune cells invasion. Expression of the fro system can be quenched by several antioxidants, such as methionine, cysteine, and others. FroR-deficient cells that lack froABCD expression during HOCl stress, appear more sensitive to the oxidant.

Strengths:

The authors provide a number of data supporting their claim that transcription of the froABCD system is induced by reactive chlorine species. This was shown by RNAseq, qRT-PCR, and through microscopy using a transcriptional reporter fusion. Likewise, elevated expression of froABCD was shown in vitro and in vivo, excluding potential in vitro artifacts. The manuscript, while mostly descriptive, is easy to follow and the data were presented clearly and convincingly. The authors have also been responsive to concerns from the previous review.

Weaknesses:

(1) Line 10: "HOCl preferentially oxidizes....". Please consider modifying the language to: "the second-order rate constant of HOCl is significantly higher with Met/Cys compared to other aa."

(2) I am not sure I completely understand Fig 1B. Is the promoter right upstream of yfp or is yfp located downstream of froA? If the latter is the case, wouldn't this be a translational fusion?

(3) My previous comment regarding why fro expression is higher during phagocytosis in macrophages compared to neutrophils has been somewhat (albeit not convincingly), addressed by the authors in the response to the reviewer, but this discussion should be part of the manuscript as the macrophage data were shown.

(4) Line 122: The statement "The degree of fro inhibition by 4-ABAH...." is incorrect unless the authors can provide experimental evidence. Fro expression is not upregulated because MPO is inhibited by 4-ABAH, which results in less hOCL production.

(5) Can Supp Fig. 1 be quantified in a similar way it was done for HOCl to allow for a better comparison if HOCl or flow is the more potent inducer?

(6) Overall, the fro expression (YFP/mCherry) seems highly variable for treatment with HOCl (Fig. 2C: ~65; 2D: ~20; why is fro expression 3x lower?

(7) The authors should provide evidence that N-chlorotaurine can activate fro expression also. They said they weren't able to obtain chlorinated taurine, but this is quite simple to produce: PMCID: PMC1219228

(8) Fig. 4 supplement 1: Please provide concentrations for the oxidants used in these experiments.

(9) Lines 251/252: change to: upregulation of instead of in

(10) Chaperones and other heat-shock genes are more upregulated in ΔfroR, indicating elevated HOCl-mediated oxidative damage, which supports their findings.

(11) Complementation of ΔfroR is missing

(12) Line 198: The growth experiment at 4 uM shows differences between WT and mutant, but at 2 uM cells showed already low fro expression due to cell death (which has not been proven by CFU counts). This discrepancy should at least be discussed.

(13) The critical in vitro experiment is missing: does purified FroI get oxidized by HOCl and dissociated from FroR?

(14) Lines: 350-355: The claim that the fro system is the first-line defense is unproven.

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Reviewer #2 (Public review):

Summary:

Foik et al. studied the regulation of the fro operon in response to HOCl, an oxidant derived from immune cells, especially neutrophils. They use a transcriptional fusion of YFP to the froA promotor in an mCherry expressing P. aeruginosa strain to determine fro-induction under the microscope. They use this system to study fro expression in medium, in the presence of neutrophils and macrophages, neutrophil-conditioned medium, and several chemical stimuli, including NaCl, HOCl, hydrogen peroxide, nitric acid, hydrochloric acid, and sodium hydroxide. They also use a corneal infection model to demonstrate that froA is upregulated in P. aeruginosa 20 h post infection and perform transcriptional analyses in WT and a froR mutant in response to HOCl.

Strengths:

Their data clearly shows that HOCl is a strong inducer of the fro Operon. Addition of HOCl-quenching chemicals together with HOCl abrogates the response. They also show that a froR mutant is more susceptible to HOCl than WT. Their transcriptomic data reveals genes under control of the FroR/FroI sigma factor/anti sigma factor system.

Weaknesses:

Although the presented evidence is mostly solid, some of their findings need to be evaluated more carefully; explaining the rationale behind some of the experiments might enhance the article; and some of the models proposed by the authors seem far-fetched, as outlined below:

Unexpected outcomes and open questions for future research:

(1) As outlined above, HOCl seems to be the main inducer of the fro operon. Interestingly, during interaction with immune cells, macrophages and neutrophils seem to induce a reporter gene under fro control in a similar manner, although macrophages are generally thought to produce less HOCl, when compared to neutrophils. May be this view needs to be revised, or another reactive species, produced by macrophages, can activate the fro operon as well.

(2) HOCl is typically unstable in the presence of biomolecules. Nevertheless, medium conditioned by activated neutrophils is a strong inducer of the fro operon. The medium used by the authors for this experiment contains taurine, and, as the authors acknowledge, this taurine will likely react with HOCl to form the more stable taurine N-chloramine. Similarly, the MinA bacterial medium used to treat P. aeruginosa with HOCl directly also contains ammonium ions at mM concentrations, which could potentially react with HOCl to form monochloramine. It could be speculated that taurine N-chloramine and other chloramines are as effective as HOCl in activating the fro-operon.

(3) The fro operon was originally described to be activated by shear stress ("flow-regulated operon"). How shear stress and HOCl-stress are related, or if fro activation by both stimuli is a coincidence, remains unclear. The authors propose a model, in which flow transports oxidizing molecules, which ultimately activate the fro operon. However, the initial work by Sanfilippo et al. (2019, Nat Microbiol) used plain LB medium in a fluidic chamber to induce the shear stress, which should be free of oxidants, and certainly of HOCl.

Comments on revised version:

The authors have addressed my concerns appropriately.

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Author response:

The following is the authors' response to the original reviews.

We greatly appreciate the efforts of the reviewers, which have provided insightful and helpful comments to improve the manuscript. The feedback touches upon a number of topics, focusing on clarification or justification of experimental techniques and on understanding the mechanism by which *P. aeruginosa* detects HOCl. All reviewers raised the issue of how HOCl activates fro expression, including whether free or protein-bound methionine, cysteine, or other HOCl byproducts induce this expression. For the upcoming revision, we plan to perform experiments that address this issue and will discuss potential mechanistic models in light of the new data. In addition, we plan to perform additional experiments to address a reviewer's concerns regarding the dependence of the *fro* response on HOCl production by neutrophils. The revision will correct imprecise statements pointed out by reviewers, and address all remaining issues requiring clarification or further discussion, including the range of HOCl sensitivity, relationship between HOCl and flow sensitivity, and justification for testing the fro response to nitric acid.

We have completed a number of experiments and responded thoroughly to reviewer comments below. We thank the reviewers again for their details comments, which suggested additional experiments and interpretations that have resulted in significant additional insight into the potential mechanism of HOCl sensing and its relevance with neutrophils.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
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> *Foik et al. report that hypochlorous acid, a reactive chlorine species generated during host defense, activates the transcription of the froABCD in P. aeruginosa. This gene cluster had previously been associated with a potential role during the flow of fluids and appears to be regulated by the sigma factor FroR and its antisigma factor FroI. In the present study, the authors show that froABCD is expressed both in neutrophils and macrophages, which they claim is likely a result of HOCl but not H2O2 production. Fro expression is also induced in a murine model of corneal infection, which is characterized by immune cell invasion. Expression of the fro system can be quenched by several*

> *antioxidants, such as methionine, cysteine, and others. FroR-deficient cells that lack froABCD expression during HOCl stress appear more sensitive to the oxidant.*
>
> *Strengths:*
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> *The authors provide a number of data supporting their claim that transcription of the froABCD system is induced by reactive chlorine species. This was shown by RNAseq, qRT-PCR, and through microscopy using a transcriptional reporter fusion. Likewise, elevated expression of froABCD was shown in vitro and in vivo, excluding potential in vitro artifacts. The manuscript, while mostly descriptive, is easy to follow, and the data were presented clearly.*

We greatly appreciate the efforts of the reviewer and thank them for their succinct summary of the manuscript.

> *Weaknesses:*
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> *(1) Lines 60-62: Some of the authors' conclusions are not supported by the data and thus appear unfounded. One example: "we determine that fro upregulation.....These data suggest a novel mechanism..." Their data do not show that MSR upregulation is a direct effect of FroABCD. Instead, it could be possible that the FroR sigma factor also controls the expression of msr genes, which would be independent of froABCD.*

We thank the reviewer for pointing out this important distinction. We have clarified in lines 63-65 in the clean version of the revision that MSR upregulation depends on FroR rather than FroABCD.

> *(2) The authors show increased fro transcription both in neutrophils and macrophages; however, the two types of immune cells differ quite dramatically with respect to myeloperoxidase activation and HOCl production.*

Neither has this been discussed nor considered here.

We agree that the distinction between the cell types is important and have added a brief description of the differences in respiratory bursts and ROS production between the two cell types and our justification for focusing on neutrophils in lines 102-103. We think it's very interesting that Fro appears to be activated by macrophages, which are not associated with HOCl production on their own. We think that it would be interesting to identify what is inducing Fro in macrophages in future work.

> *(3) With respect to the activation of fro expression upon challenge with conditioned media from stimulated neutrophils, does the conditioned media contain detectable amounts of HOCl? Do chloramines, which are byproducts of HOCl oxidation with amines, also stimulate expression?*

This is an excellent question that addresses which molecules Fro is responding to from neutrophils. We have performed additional experiments that confirm that PMA-stimulated neutrophils produce HOCl (Fig. S2) through the use of a commercial hypochlorite sensor assay, which claims high specificity for detecting HOCl. We further confirmed that this production is inhibited by pretreatment with the MPO-specific inhibitor 4ABAH. These data support the interpretation that the Fro response to stimulated neutrophils requires MPO activity, of which the major product is HOCl. We have described this in lines 117-127.

Our data does not exclude the possibility that other MPO products could activate Fro expression. We were unable to obtain a reliable source of the major secondary MPO product, taurine chloramine, for our experiments, unfortunately. We believe that understanding the potential for secondary products to activate the response is an important and interesting

question that can be explored in a future study. We have added a discussion of this in lines 367-376.

> *(4) A better control to prove that this fro expression is indeed induced by HOCl in activated neutrophils would be to conduct the experiments in the presence of a myeloperoxidase inhibitor.*

We thank the reviewer for raising this point. We have performed the suggested set of experiments and found that indeed, the pre-treatment of neutrophils with MPO inhibitor 4-ABAH prior to PMA stimulation suppresses the activation of *fro* (Fig. 2D and Figure 2-figure supplement 1-2). The results are discussed in lines 117-127.

> *(5) The work was conducted with two different P. aeruginosa strains (i.e. AL143 and PAO1F). None of the figure legends provides details on which strain was used. For instance, in line 111, the authors refer to Figure S1B for data that I thought were done with PAO1F, while in 154, data were presented in the context of the infection model, which was conducted with the other strain.*

We thank the reviewer for pointing this issue out. We have ensured that strain names appear in all the revised figure legends. To clarify, only mouse experiments and a related RT-qPCR assay used strain PAO1F due to prior IACUC approval of this strain and its use in previous publications.

> *(6) It would be good if immune cell recruitment at 2hrs and 20hrs PI could be quantified.*

We previously quantified neutrophil recruitment at the site of corneal abrasion at 24 hours using the same conditions and strains (Ratitong, B. et al., J. Immun, 2022). While we do not have immune cell recruitment data for the 2 hr and 20 hr time points, the previous data show significant neutrophil recruitment near the latter time point, which is consistent with the interpretation that *fro* expression is activated by stimulated neutrophils. We have discussed this in lines 212-216.

> *(7) The conclusions of Figure 4 are, in my opinion, weak (line 187-188; "It is possible that ....."). These antioxidants likely quench the low amounts of NaOCl directly. This would significantly reduce the NaOCl concentrations to a level that no longer activates expression of fro. There is no direct evidence provided that oxidized methionine induces fro expression. Do the authors postulate that this is free methionine, or could methionine and/or cysteine oxidation in FroR increase the binding affinity of the sigma factor to the promoter? Another possibility is that NaOCl deactivates the anti-sigma factor. None of these scenarios has been considered here.*

We acknowledge that our model of HOCl sensing was unclear and thank the reviewer for their insight. This critique is echoed by reviewer #2 in comment 3 as well. We recently found that the FroI anti-sigma factor has the highest concentration of methionine and cysteine residues of all known *P. aeruginosa* anti-sigma factors (Appendix 2—Table 1). Given that FroR and FroI form an extracytoplasmic function sigma – anti-sigma pair, which are associated with transducing extracellular signals to the cytoplasm, we have proposed an alternative model in which HOCl or secondary RCS molecules are detected through their oxidation of cysteine and methionine residues in FroI. This is discussed in lines 271-279 and lines 363-367.

> *(8) Line 184: The reaction constants of HOCl with Cys and Met are similar.*

We thank the reviewer for pointing out this important clarification. We have revised the sentence to accurately reflect this in lines 243-244.

> *(9) Treatment with 16 uM NaOCl caused a growth arrest of ~15 hrs in the WT (Figure 5A), whereas no growth at all was recorded with 7.5 uM in Figure 3A.*

We thank the reviewer for catching this. We have determined that the concentration of NaOCl in the reagent used for this particular experiment was lower than expected, thus requiring a higher concentration to achieve growth inhibition. We have repeated the experiment with new reagent and find that the results (now Figure 6A) are similar to the previous experiment but at a lower concentration of 4 micromolar, consistent with the concentration found to be sub-inhibitory in Figure 3A.

> *(10) The concentration range of NaOCl causing fro expression is extremely narrow, while oxidative burst rapidly generates HOCl at much higher concentrations. This should be discussed in more detail.*

We appreciate the reviewer's comment, which is related to reviewer #2's comment #9. We have clarified the reported production rates of HOCl, which far surpass the bacterial MIC. After greater consideration, we believe secondary HOCl products including taurine chloramine could have a more significant role *in vivo*. While this molecule is less potent than HOCl, it is longer-lived, retains bactericidal activity, and retains the ability to oxidize methionine. We have discussed this in lines 377-405.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Some statements in the text don't match the data shown in the Figures. For instance:*
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> *(a) Figure 2B shows ~65-fold fro expression, but the text states: "...increased expression of fro expression by 30fold..."(line 88).*

The YFP/mCherry value of PMA-stimulated is 67.4 in the Figure (now Figure 2C). The fold-change is computed relative to unstimulated conditioned medium (third column, which has a value of 2.2), which is a 30-fold change. We have added a citation in the main text to the Source Data, which provides these raw values, to help clarify the computation for readers, and added in the legend that the value for unstimulated is greater than 1.

> *(b) Figure 3B shows ~35-fold fro expression at 1 uM NaOCl, but the text states: "...NaOCl increased fro expression by up to 74-fold..."(line 110).*

We have clarified that the increase is relative to untreated, added that untreated value is below 1 in the caption, and provided a citation in the main text to the Source Data, which contains the raw values. The change is measured relative to untreated, for which the YFP/mCherry value is 0.48. The value at 1 uM is 35.7, giving a 74-fold change.

> *(2) Line 229: While the ΔfroR strain was sensitive to HOCl, the strain was tolerant". Please revise.*

We have corrected this typo (now lines 328- 329). We meant to convey that growth was not entirely inhibited in the froR strain.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Foik et al. studied the regulation of the fro operon in response to HOCl, an oxidant derived from immune cells, especially neutrophils. They use a transcriptional fusion of YFP to the froA promoter in an mCherry-expressing P. aeruginosa strain to determine fro-induction under the microscope. They use this system to study fro expression in medium, in the presence of neutrophils and macrophages, neutrophil-conditioned medium, and several chemical stimuli, including NaCl, HOCl, hydrogen peroxide, nitric acid, hydrochloric acid, and sodium hydroxide. They also use a corneal infection model*

> *to demonstrate that froA is upregulated in P. aeruginosa 20 h post-infection and perform transcriptional analyses in WT and a froR mutant in response to HOCl.*
>
> *Strengths:*
>
> *Their data clearly shows that HOCl is a strong inducer of the fro Operon. The addition of HOClquenching chemicals together with HOCl abrogates the response. They also show that a froR mutant is more susceptible to HOCl than WT. Their transcriptomic data reveal genes under control of the FroR/FroI sigma factor/anti sigma factor system.*
>
> *Weaknesses:*
>
> *Although the presented evidence is mostly solid, some of their findings need to be evaluated more carefully; explaining the rationale behind some of the experiments might enhance the article, and some of the models proposed by the authors seem far-fetched, as outlined below:*

We greatly appreciate the reviewer's efforts and thank them for highlighting strengths and areas for improvement.

> *(1) In line 76 the authors claim "Relative to P. aeruginosa that were incubated in host cell-free media, P. aeruginosa in close proximity to human neutrophils or that were engulfed in mouse macrophages appeared to increase fro expression (Fig. 1C)". Counting bacterial cells in Figure 1C shows that 1 in 17 bacteria (5.8%) induce the froA-promotor in media in the absence of immune cells, while 4 in 72 bacteria (only 5.5%) do the same in the presence of neutrophils. Contrary to the authors' claims, it appears that P. aeruginosa actually decreases fro-expression in close proximity to neutrophils. There is a slight increase in fro-expression in bacteria co-incubated with macrophages (3 in 21, or 14.3%). A more rigorous statistical analysis might substantiate the authors' claim, but, as is, the claim "neutrophils increase fro expression" is untenable.*

We believe the images alone do not give an adequate representation of the data and have quantified a larger portion of the data, which has been added as Figure 1D. The quantification supports the original claim that *fro* expression is increased during co-incubation with macrophages and neutrophils. Since there was not sufficient statistical sampling to distinguish engulfed *P. aeruginosa* from free ones, this part of the claim has been removed from the text (updated in lines 80-84).

> *(2) The authors should explain the rationale behind some of the chemicals used. Why did they use nitric acid? Especially at these high concentrations, a strong acid such as nitric acid might have a significant influence on the medium pH. I understand that the medium is phosphate-buffered, but 25 mM nitric acid in an unbuffered medium would shift the pH well below 2. Similar considerations apply to hydrochloric acid and sodium hydroxide.*

We thank the reviewers for pointing out the need for this clarification. We have updated Figure 3D with a lower concentration of NaOH at 1 uM, which is the same concentration as NaOCl that activates *fro* expression. Due to the high buffering capacity of our medium, a high concentration of 6 mM NaOH was needed to induce a discernible change in pH and this high concentration of NaOH had no obvious effect on growth. Neither 1 uM nor 6 mM NaOH produced a change in *fro* expression, consistent with our previous findings that the effect is not due to sodium ions or higher pH. These updated findings are described in lines 181-190.

Since the effect of chloride is already controlled for using NaCl and the concentration of HCl used was not sufficient to cause a significant change in pH in the buffered medium, we have removed the HCl group from the data.

We have clarified that nitric acid was used because it is a strong oxidizer that is not found in neutrophils and that concentrations used were near the minimal inhibitory concentrations (lines 145-147 and lines 191-198). We acknowledge that the growth inhibition from HNO_3 could be due to pH or oxidation. However, since no change in *fro* expression was observed at concentrations approaching the inhibitory concentration, we did not address the potential effects of low pH from nitric acid on *fro* expression.

> *(3) In line 187, the authors state that "It is possible that oxidized methionine increases fro expression" and they suggest a model to that effect in Figure 5D. It is unclear why the authors singled out methionine sulfoxide, since a number of other things get oxidized by HOCl. In line 184, the authors state, in the same vein, that "HOCl oxidizes methionine residues 100-fold more rapidly than other cellular components". The authors should state which other cellular compounds they are referring to. Certainly not cysteine and other thiols, which react equally fast and are highly abundant in the cell: P. aeruginosa contains 340 μM GSH, 140 μM CoA-SH (https://doi.org/10.1074/jbc.RA119.009934) plus free cysteine and cysteines in proteins (based on codon usage, 1.34% of amino acids in proteins are cysteine, while methionine is only slightly more present at 2.10%, although a number of starting methionines are removed from mature proteins).*

We acknowledge that our HOCl sensing model had been vague and unclear and thank the reviewer for their insight. This critique is echoed by reviewer #1 in comment 7 as well.

Our initial suggestion that methionine sulfoxide was sensed was motivated by the observation that methionine sulfoxide reductases are upregulated by HOCl. However, we have revised this based on feedback from reviewers and further consideration of chlorine redox chemistry. Interestingly, we found that the FroI anti-sigma factor has the highest concentration of methionine and cysteine residues of all the *P. aeruginosa* anti-sigma factors (Appendix 2—table 1). Given that FroR and FroI form an extracytoplasmic function sigma – anti-sigma pair, which are associated with transducing extracellular signals to the cytoplasm, we propose a model in which HOCl or secondary RCS molecules are detected by their oxidation of cysteine and methionine residues in FroI. This is discussed in lines 271-278 and lines 362-366.

> *(4) Overall (and this is probably not addressable with the authors' data), some very interesting questions remain unanswered: what is the molecular mechanism of fro-induction? How is the FroR/FroI system modulated by HOCl? Does the system sense free or protein-bound methionine-sulfoxide? Are certain methionine residues in these proteins directly oxidized by HOCl? Many "HOCl-sensing" proteins are also modified at cysteine residues or amino groups; could those play a role? And lastly: what is the connection between shear/fluid flow and HOCl, or are these totally separate mechanisms of fro-induction?*

We thank the reviewer for raising these excellent mechanistic questions. Issues relating to HOCl sensing are addressed in the preceding comment.

Regarding the connection to shear sensing, Padron et al., 2023 found that the detection of flow in *P. aeruginosa* can be attributed to chemical transport, in particular to H_2O_2 that was present in growth media. Based on the same principle, we expect Fro to be upregulated in flow at much lower concentrations than those observed in stationary fluids. The activation of Fro and the effects of HOCl would thus be expected to be flow-sensitive. We have commented on this important factor in the discussion in lines 406-417.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) To address 1, the authors could evaluate the microscopic images in the same manner in which they evaluated the other microscopic images, as, for example, presented in*

> *Figure 2B or Figure S1B.*

See response to Weakness point (1).

> *(2) To address 2, please explain the choice of the chemicals (why nitric acid?), but also provide the pH of the media with those high concentrations of strong acids and bases, and interpret them in light of the permissible pH range for P. aeruginosa growth. More sensible controls might be a lower NaOH concentration in the range that would be reached through the amount of NaOH in the NaOCl stock at the highest NaOCl concentrations used. As for the acids, I don't see a reason to use these acids at these high concentrations. Please explain.*

See response to Weakness point (2).

> *(3) To address 3: The authors could specify their methionine-sulfoxide model a bit more, so that testable hypotheses can be developed. If the authors think the FroR/FroI system senses free methionine sulfoxide, they or others could add methionine sulfoxide to the medium and check induction. If they think specific methionine residues in these proteins are oxidized, they could provide evolutionary evidence of conserved methionine residues. Or, based on a structure or structural prediction, they (or others) could mutate methionine residues, e.g., at the protein's surface or potential protein/protein-interaction sites and assess the effect on HOCl-based activation. Or they could consider other amino acids known to be highly reactive towards HOCl and mutate those in a future study.*

See response to Weakness point (3).

> *Further comments:*
>
> *(4) The headline of the figure legend of Figure S1 seems incomplete. Please mention the flow experiments shown in Figure S1A.*

We have updated the title of this figure, which now appears in the eLife format as Figure 1 – figure supplement 1.

> *(5) What is the difference between the data presented in Figure 4C (bars "UTR" and "NaOCl") and the same bars in Figure S2A? Is this redundant or a re-plot?*

In this revision, Figure 4C has become Figure 5B and Figure S2A is now Figure 5—figure supplement 1. Only the 1 uM NaOCl condition is replotted. We have described this in the legend for Figure 5—figure supplement 1.

> *(6) Line 228: hpd is more likely a gene of the aromatic amino acid catabolism.*

We thank the reviewer for pointing this out. We have removed the 'branched' descriptor in this sentence, now in lines 325-326.

> *(7) Line 229: "While the ΔfroR strain was sensitive to HOCl, the strain was tolerant (Figure 5A)". Please clarify. Which strain was tolerant? WT?*

We have corrected this typo (now line 328-329). We meant to convey that growth was not entirely inhibited in the froR strain.

> *(8) Line 247: "Activated neutrophils produce HOCl concentrations as high as 50 μM [24]." This "50 μM" number is often quoted; the citation trail typically leads to Weiss et al. 1982 (https://doi.org/10.1172/JCI110652). However, a more factually correct statement based on that paper would be "2 x 10^6 neutrophils, activated with 30 ng/mL PMA at 37C in 1 mL of Dulbecco's buffer can produce around 50 nmol HOCl per hour". In the particular reference 24, Dybpukt et al used a methodology similar to Weiss et al., and*

> *here around 50nmol were produced by the same number of cells in 30 min in response to 100 ng/mL PMA. Please clarify accordingly.*

We thank the reviewer for bringing this to our attention. We have altered the language to indicate that the production rate is for this specific set of parameters. Related to this, Reviewer #1 Comment #10 requested a more detailed discussion of the significance of the Fro response, since it is at much lower HOCl concentration than produced by neutrophils. We have discussed this in lines 377-405.

> *(9) Line 275: "which is strain PA14 strain". Please clarify.*

We have fixed this error and entered it into the Key Resource Table.

> *(10) Line 311: "Cultures containing densities below 10 P. aeruginosa per frame were concentrated using a syringe filter with 0.2 or 0.8 µm pore sizes (Millipore, Burlington, MA)." Isn't that a bit concerning when testing the induction of an operon that is supposedly activated by shear through fluid flow? Did the authors convince themselves that this procedure does not induce fro?*

We do not expect the filtering procedure to cause changes in gene expression because cells are imaged immediately after filtering. Nonetheless, we performed additional experiments (Fig 3F and 2D) entirely without concentrating cells and found that YFP/mCherry levels were consistent with previous data in Figure 3. This rationale has been added to the Methods section under the “Fluorescence and Phase Contrast Microscopy” section.

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