The Diguanylate Cyclase YfiN of *Pseudomonas aeruginosa* Regulates Biofilm Maintenance in Response to Peroxide

Stefan Katharios-Lanwermeier, Sophia A. Koval[†], Kaitlyn E. Barrack[†] and G.A. O'Toole*

Dept. of Microbiology & Immunology, Geisel School of Medicine at Dartmouth, Hanover, NH

[†]These authors contributed equally to this work.

*To whom correspondence should be addressed:

Rm 202 Remsen Building

66 College Street

Geisel School of Medicine at Dartmouth

Hanover, NH 03755

Phone: (603) 650-1248 (office)

email: georgeo@Dartmouth.edu

Running title: YfiN Regulates Biofilm Maintenance

Key words: biofilm, c-di-GMP, peroxide, diguanylate cyclase, *Pseudomonas aeruginosa*

Abstract

Pseudomonas aeruginosa forms surface-attached communities that persist in the face of antimicrobial agents and environmental perturbation. Published work has found extracellular polysaccharide (EPS) production, regulation of motility and induction of stress response pathways as contributing to biofilm tolerance during such insults. However, little is known regarding the mechanism(s) whereby biofilm maintenance is regulated when exposed to such environmental challenges. Here, we provide evidence that the diguanylate cyclase YfiN is important for the regulation of biofilm maintenance when exposed to peroxide. We find that, compared to the wild type (WT), static biofilms of the $\Delta yfiN$ mutant exhibit a maintenance defect, which can be further exacerbated by exposure to peroxide (H_2O_2); this defect can be rescued through genetic complementation. Additionally, we found that the $\Delta yfiN$ mutant biofilms produce less c-di-GMP than WT, and that H_2O_2 treatment enhanced motility of surface-associated bacteria and increased cell death for the $\Delta yfiN$ mutant grown as a biofilm compared to WT biofilms. These data provide evidence that YfiN is required for biofilm maintenance by *P. aeruginosa*, via c-di-GMP signaling, to limit motility and protect viability in response to peroxide stress. These findings add to the growing recognition that biofilm maintenance by *P. aeruginosa* is an actively regulated process that is controlled, at least in part, by the wide array of c-di-GMP metabolizing enzymes found in this microbe.

Importance

We build on previous findings that suggest that *P. aeruginosa* utilizes c-di-GMP metabolizing enzymes to actively maintain a mature biofilm. Here, we explore how the diguanylate cyclase YfiN contributes to the regulation of biofilm maintenance during peroxide

exposure. We find that mature *P. aeruginosa* biofilms require YfiN to synthesize c-di-GMP, regulate motility and to insure viability during peroxide stress. These findings provide further evidence that the modulation of c-di-GMP in response to environmental signals is an important mechanism by which biofilms are maintained.

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that is found in settings as varied as natural aquatic environments, implanted catheters and the airways of patients afflicted with cystic fibrosis (1–3). *P. aeruginosa* forms robust biofilms, multicellular surface-attached aggregates that are recalcitrant to antimicrobial therapy and environmental insults (4, 5). For motile organisms such as *P. aeruginosa*, biofilms form through a multistep process that requires motility and motility appendages, surface-sensing and the production of extracellular polysaccharides (EPS) (6–9). Flagella mediate initial polar attachment of a cell to a surface, while pili facilitate irreversible surface attachment and microcolony formation (10, 11). After surface attachment, the PilY1 protein is secreted to the cell surface where it contributes to surface sensing and the activation of diguanylate cyclase SadC via a Type IV pili (TFP) dependent, outside in signal transduction pathway (8, 12). The activation of SadC results in increased bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (12), a second messenger that coordinates surface behavior, virulence and the production of EPS (13–16). Concentrations of c-di-GMP are controlled enzymatically by diguanylate cyclases that synthesize this molecule and phosphodiesterases that degrade this second messenger (17–19). c-di-GMP controls events in biofilm formation beyond surface attachment. For example, once attached to a surface, the production of EPS, a process also regulated by c-di-GMP, provides microcolonies

with architectural strength for continued growth and protection against predation and environmental challenges, such as antimicrobials, oxidative and osmotic stressors (4, 20–24).

Biofilms provide protection from a number of insults, including peroxide stress. For example, *Acinetobacter oleivorans* and *Helicobacter pylori* (25, 26) both respond to peroxides with increased EPS production and enhanced biofilm biomass, while for *P. aeruginosa* the peroxide (H₂O₂)-responsive OxyR regulates metabolism through the control of iron homeostasis and protein synthesis (27). However, little is known regarding how mature biofilms respond to the exposure to peroxides, including H₂O₂, an important issue given that molecules with such chemistry are produced by the immune system (3, 28, 29).

While early surface attachment events and the processes that drive biofilm maturation (i.e., reversible/irreversible attachment, microcolony formation, EPS production) have received a great deal of attention in the literature, less is known about the regulatory components needed for biofilm maintenance. In previous work in a collaborative effort with the Howell group, we found that RmcA and MorA, phosphodiesterases that break down c-di-GMP, regulate the maintenance of nutrient-limited biofilms (30). To assess if genes that encode diguanylate cyclase activity also contribute to biofilm maintenance, we utilized a mutant library containing clean deletions of all known single-domain DGCs (31). We screened mutants that individually lacked these DGC domains in a static assay to identify deficits in biofilm maintenance. Through this process, we identified YfiN (32) as a putative biofilm maintenance protein. The *yfiN* gene has previously been found to be a member of the *yfiBNR* operon (33). Repression of YfiN activity is mediated by its regulator, YfiR. YfiR activity is repressed when bound by YfiB under some conditions (33, 34). Oxidative stress is thought to play an important role in the regulation of YfiBNR activity, with the crystal structures of YfiR indicating denaturation of this protein in oxidizing

conditions (35) Here, we investigate the role of YfiN in *P. aeruginosa* motility and biofilm maintenance, and how peroxide stress impacts these biological functions.

Results

The $\Delta yfiN$ mutant is defective for biofilm maintenance and susceptible to peroxide stress.

We previously reported the use of a library of in-frame deletion mutants targeting each of ~40 genes of *P. aeruginosa* PA14 predicted to participate in c-di-GMP synthesis or degradation (31). We used this library to determine if any of the genes that encode diguanylate cyclases (DGCs) contribute to the ability of *P. aeruginosa* to maintain a biofilm once the community has been established. To test this idea, strains carrying mutations in each of the DGCs of *P. aeruginosa* PA14 were grown statically in 96-well plates for 12h and 48h in M63 biofilm medium (**Table S1**). As we have described previously, the identification of candidate mutants involved in biofilm maintenance is apparent through a biofilm defect at 48h, but not at 12h (30). From our initial screen of these DGC mutants, we found that the strain carrying a mutation in the *yfiN* gene exhibited a biofilm comparable to WT at 12h (**Fig. 1A**, left panel) but displayed a significant defect at 48h, with lower amounts of biofilm biomass at this time point (**Fig. 1A**, middle panel).

To verify that the loss of the biofilm that we observed at the 48h assay was dependent on the absence of the *yfiN* gene, we genetically complemented the $\Delta yfiN$ mutant with a plasmid generously provided by Thomas Wood (32). After induction with arabinose (0.2% w/v) to induce expression of this gene from the plasmid, we found that biofilms of WT carrying the pMQ70 empty vector (EV), the $\Delta yfiN$ mutant carrying the EV, and $\Delta yfiN$ mutant complemented with pMQ70-*yfiN* to be comparable at 12h (**Fig. 1A**, left panel). The biofilm defect at 48h observed in

the $\Delta yfiN$ mutant carrying the EV can be largely rescued for the $\Delta yfiN$ mutant complemented with pMQ70-*yfiN* (Fig. 1A, right panel).

PAO1#	PA14#
0169 (<i>siaD</i>)	02110
0290	03790
0338	04420
3702 (<i>wspR</i>)	16500
3343 (<i>hsbD</i>)	20820
3177	23130
2870	26970
1851	40570
1120 (<i>yfiN/tpbB</i>)	49890
1107 (<i>roeA</i>)	50060
0847	53310
4332 (<i>sadC</i>)	56280
4396	57140
4843 (<i>gcbA</i>)	64050
4929	65090
5487 (<i>dgcH</i>)	72420

Table S1. Diguanilate cyclase mutants screened for defects in biofilm maintenance.

Previous work by Ha et al. (31) created in-frame, unmarked deletions of all putative diguanilate cyclases that were identified through SMART analyses of proteins encoded in the *P. aeruginosa* genome (31). Here, these mutants were grown statically in 96-well plates for 12h and 48h in M63 biofilm medium. Of all mutants screened, only the $\Delta yfiN$ mutant (red) exhibited both comparable biofilm biomass to the WT at 12h, yet was unable to maintain a biofilm at 48h.

We next wanted to understand if the observed biofilm defect of the $\Delta yfiN$ mutant at 48h was elicited by starvation or other consequences of prolonged batch culture growth. To test this idea, we repeated the 48h static biofilm assay but periodically (every 12h) removed the spent medium, washed the biofilm and added fresh medium. Through this medium replacement assay, we observed that the biofilm defect of the $\Delta yfiN$ mutant was rescued at 48h (Fig. 1B, right panel), with the level of biofilm staining comparable to the WT (Fig. 1B, left panel).

Figure 1. The $\Delta yfiN$ mutant is defective for biofilm maintenance and a pre-formed biofilm is reduced when exposed to peroxide. A) Biofilm formation by the WT, the WT carrying the pMQ70 empty vector (EV), the $\Delta yfiN$ mutant carrying the EV, and $\Delta yfiN$ mutant complemented with pMQ70- $yfiN$ after 12h and 48h (left and right panel, respectively). The biofilm formed was assessed by staining with crystal violet (CV), the CV-stained biofilm solubilized in 30% glacial acetic acid and the absorbance (OD₅₅₀) measured. B) Biofilm formation by WT and the $\Delta yfiN$ mutant was assessed in a 96-well plate assay. The strains were grown on M63 medium supplemented with arginine (0.4% w/v) and the biofilm measured after 12h (left panel), 48h (middle panel), or after 48h wherein the medium was replaced every 12h (right panel). The quantification of the biofilm by CV was performed as described in panel A. C) Biofilms of WT and the $\Delta yfiN$ mutant were grown for 12h, after which medium was replaced with control growth medium (left panel) or medium supplemented with 50 mM H₂O₂ (+H₂O₂) for an additional 12h of growth. Biofilm formation was assessed as described in panel A. For all panels, error bars represent standard deviation of the results from three biological replicates each performed with three technical replicates. Statistical significance was assessed via Student's T-test in all panels except 1A, in which Dunnett's multiple comparisons post-test was used. *, **, indicate differences that are significantly different at P<0.05 and P< 0.01, respectively, compared to the WT. ns; non-significant.

Considering the observed biofilm maintenance defect of the $yfiN$ null mutant and the previous reports indicating that YfiR activity is regulated by oxidative stress (35), we

hypothesized that the biofilm maintenance defect manifested by the $\Delta yfiN$ mutant may be due to an altered response to peroxide stress. To test this hypothesis, we compared the amount of biofilm retained by WT or the $\Delta yfiN$ mutant after 24h with a single medium replacement at 12h, to biofilms of these same strains exposed to 50 mM H_2O_2 added to the replacement medium. After 24h with control replacement medium, biofilms of WT and the $\Delta yfiN$ mutant were not significantly different (**Fig. 1C**, left panel). However, when replacement medium contained 50 mM H_2O_2 we observed that biofilms of the $\Delta yfiN$ mutant exhibited a significantly lower biofilm biomass compared to the WT (**Fig. 1C**, right panel). Collectively, these results suggest that YfiN may be important for biofilm maintenance and that *P. aeruginosa* biofilms lacking this DGC exhibited reduced biofilm biomass when exposed to peroxide.

Dispersion and cell death contribute to the $\Delta yfiN$ mutant biofilm maintenance defect. The above results provide evidence that YfiN impacts biofilm maintenance in the face of peroxide stress. In order to determine biomass and cell viability before and after treatment, static biofilms were grown in glass-well dishes containing the same medium used in the 96-well dish assay to directly image the biofilms. The biofilm biomass was determined by measuring the fluorescence of the WT and the $\Delta yfiN$ mutant strain carrying the multicopy plasmid pSMC21, which constitutively expresses GFP (ex500/em513). To visualize dead cells, the biofilms were washed with the same culture medium containing propidium iodide (PI) immediately prior to imaging. Fluorescence intensity was used to quantify the biofilm biomass of each strain (green fluorescence), and the ratio of PI to GFP intensity was used as a metric to measure normalized cell death within the biofilms.

Here, the $\Delta yfiN$ mutant exhibits a mild, but in this case significant, defect in the biofilm biomass at 12h compared to the WT in this glass dish assay (**Fig. 2A, E-F**). These data suggest that both strains can establish a biofilm. Consistent with the 96-well dish assay (**Fig. 1A**), the $\Delta yfiN$ mutant shows significantly less biofilm biomass at 24h compared to the WT (**Fig 2B, G, H**). Thus, we can use the glass bottom dishes to visualize the biofilms and largely replicate the findings observed in the 96-well dish assay.

When we assessed cell viability via the ratio of PI-stained dead cells to GFP-labeled live cells, we observed no difference between the WT and $\Delta yfiN$ mutant at 12h (**Fig. 2C**). While we did detect dead (PI-stained) cells for the WT strain at 24h, the extent of cell killing as assessed by the PI/GFP ratio was significantly higher for the $\Delta yfiN$ mutant (**Fig. 2D**). Representative images used to generate the quantitative data in panels A-D are shown in **Fig. 2** panels E-H. Together, these data suggest that the loss of the biofilm in the $yfiN$ mutant at 24h is due, at least in part, to increased cell death in this mutant.

Figure 2. Cell death likely contributes to the $\Delta yfiN$ mutant biofilm maintenance defect.

Biofilm biomass and cell death was assessed microscopically in glass-bottom dishes with WT and the $\Delta yfiN$ mutant. Cell biomass was determined using fluorescence mediated by the plasmid pSMC21, which encodes a constitutively-expressed GFP. Cell viability was determined as a ratio of propidium iodide (PI)-stained (dead) cells to GFP-mediated green fluorescence (live cells). The PI was added to the dishes immediately prior to visualizing, and the cells were washed to remove excess dye. A-B) WT and the $\Delta yfiN$ mutant biofilm biomass was quantified by

measuring GFP intensity at 12h (A) and 24h (B). We observed that the biofilm maintenance defect of the $\Delta yfiN$ mutant occurred as early as 24h in the glass-bottom plates assay and consequently used this point for the analysis here. C-D) Cell death was measured as a ratio of PI staining to GFP intensity at 12h (C) and 24h (D). For all panels, error bars represent standard deviations of the results from three biological replicates each performed with 6 technical replicates. Statistical significance was assessed via a Student's T-test. **, ***, indicate differences that are significantly different at $P < 0.01$ and $P < 0.001$ respectively, compared to the WT. ns, non-significant. (E-H) Representative images showing combined GFP (blue) and PI staining (pink) of WT biofilm at 12h (E) and 24h (G), and the $\Delta yfiN$ mutant biofilm at 12h (F) and 24h (H).

The $\Delta yfiN$ mutant biofilm exhibits increased susceptibility to peroxide stress under flow.

The static biofilm data presented thus far are consistent with the hypothesis that YfiN contributes to biofilm maintenance and that the loss of this DGC enhances cell death as these communities age and/or encounter oxidative stress in the form of peroxide. We next wanted to determine the impact of H_2O_2 specifically on the $\Delta yfiN$ mutant biofilm under flow conditions, which helps mitigate the presence of potential confounding factors, such as accumulating metabolites in the extracellular milieu. To address this question, we pursued a microfluidics-based approach in which WT and the $\Delta yfiN$ mutant with constitutive expression of GFP (using pSMC21, described above) were grown as a biofilm under flow in biofilm medium, a buffered minimal medium containing 1.0 mM K_2HPO_4 , 0.6 mM $MgSO_4$ and 0.4% arginine, pH=7.5. This medium is similar to the M63-based medium used in the static assay but has been optimized for use in flow studies.

First, to determine if the loss of YfiN adversely impacted the ability of this mutant to form a biofilm under standard flow conditions (i.e., no H_2O_2 added), we compared GFP intensity with that of WT over a 12h time course kinetic assay. The WT and $\Delta yfiN$ mutant biofilms were comparable for the first 10h of this assay, after which WT biofilm was modestly but significantly higher than that of $\Delta yfiN$ mutant in three of the last five time points assessed after 10h (**Fig. S1**).

225

226 **Figure S1. Initial biofilm formation by WT and the $\Delta yfiN$ mutant under flow.** The WT strain
227 and the $\Delta yfiN$ mutant carrying pSMC21, a plasmid which constitutively expresses GFP, were
228 inoculated into microfluidic chambers and allowed 1h to attach prior to beginning flow of
229 biofilm medium. Flow was resumed, then GFP fluorescence was measured every 20 minutes for
230 12h. Error bars represent standard deviations of the results from three biological replicates each
231 performed with three technical replicates. Statistical significance was assessed via Sidak's
232 multiple comparisons post-test. The WT formed significantly more biofilm at 10.7, 11 and 11.7h.

233 **Figure 3. The $\Delta yfiN$ mutant biofilm exhibits increased cell death and dispersal in response**
234 **to peroxide treatment.** A) Biofilm formation by the WT and the $\Delta yfiN$ mutant was quantified
235 through fluorescent microscopy and GFP signal intensity measured for 10h after the addition of
236 H_2O_2 . B) Cell death was measured as a ratio of PI to GFP over the same time course as panel A.
237 C) WT and the $\Delta yfiN$ mutant carrying plasmid pSMC21 encoding GFP were inoculated into a
238 microfluidics device and grown in biofilm medium. After 14h of growth, medium was replaced
239 with biofilm medium containing 10 mM H_2O_2 and PI. Bacterial CFUs/mL were measured in the

effluent 30 min prior to and up to 180 min after being exposed to H₂O₂. Error bars represent standard deviations of the results from three biological replicates each performed with three technical replicates. Statistical significance was assessed via Sidak's multiple comparisons post-test. Compared to WT, results from the $\Delta yfiN$ mutant were significantly different ($P < 0.05$) from WT after 6h in Figure 3A, 3h in Figure 3B, and at 45 min and 120 min in Figure 3C. D) Representative images from microfluidics studies used to generate the quantitative data presented in panels A and B. Viable cells (GFP) are colored blue while PI-stained cells are colored magenta.

We first assessed whether the $\Delta yfiN$ mutant was more susceptible to starvation than the WT, as was reported for strains carrying mutations in the *rmcA* or *morA* genes (30); we observed no such phenotype in the microfluidic devices (not shown). To determine whether the $\Delta yfiN$ mutant was more susceptible to H₂O₂ under flow compared to the WT, both strains were grown for 14h as a biofilm, after which influent biofilm medium was replaced with medium containing 10 mM H₂O₂, as well as PI to stain dead cells, and the biofilms imaged over an additional 12h time course. We then assessed biofilm biomass, cell viability and cell dispersion after this 12h exposure to peroxide.

Using GFP signal intensity as a measure of biofilm biomass, we found that prior to the addition of H₂O₂, there was no significant difference in the biofilm biomass of the WT versus the $\Delta yfiN$ mutant over the first 10h of the experiment (**Fig. S1**), indicating that both strains could form a robust biofilm. We did note a difference in the biofilm biomass between these strains under flow after 12h, but this reduction was modest and not significant at every time point (see **Fig. S1**). Monitoring the biofilm biomass showed that the addition of H₂O₂ resulted in a significant decrease in $\Delta yfiN$ biofilm biomass 6h after treatment compared to the WT, which continued to increase in biomass as judged by GFP signal intensity (**Fig. 3A, D**). Our analysis shows that H₂O₂ treatment adversely impacted the viability of $\Delta yfiN$ mutant; this mutant

exhibited a significant increase in its PI/GFP ratio compared to WT starting 3h after H₂O₂ treatment and at each subsequent time point (**Fig. 3B, D**).

To determine if H₂O₂ exposure caused cells to disperse from the biofilm, colony forming units (CFUs) were measured in the effluent 30 min prior to and for 3h after the H₂O₂-containing medium switch. Prior to exposure to H₂O₂, cell dispersal between WT and mutant $\Delta yfiN$ were not significantly different. However, the switch to medium containing 10mM H₂O₂ resulted in modest increases of $\Delta yfiN$ mutant cells in the effluent, and this difference was significant at 45 and 120 min post-stress exposure (**Fig. 3C**). Collectively, the use of static and microfluidics assays showed that the loss of YfiN function resulted in increased cell death, as well as a modest increase in dispersal of the biofilm cells compared to the WT.

To test if the enhanced H₂O₂ sensitivity exhibited by the $\Delta yfiN$ mutant was specific to the biofilm lifestyle, we washed and resuspended overnight-grown planktonic cultures of the WT and the $\Delta yfiN$ mutant and challenged these strains with 10mM H₂O₂ for 4 hours. There was no significant difference between the viability of the WT and the $\Delta yfiN$ mutant (**Fig. S2**), indicating the sensitivity to H₂O₂ was biofilm-specific.

Figure S2. The $\Delta yfiN$ mutant does not exhibit a significant increase in H₂O₂ susceptibility during planktonic culture. The WT strain and the $\Delta yfiN$ mutant were inoculated into biofilm medium, grown for 24h, washed, re-suspended in fresh medium with and without 10mM H₂O₂ to

a normalized OD₆₀₀ of 0.5. CFU/mL were measured prior to and 2-4h after the addition of H₂O₂. Error bars represent standard deviation of the results from three biological replicates each performed with three technical replicates. Statistical significance was assessed with a Sidak's multiple comparisons post-test; ns, indicates a non-significant.

Loss of YfiN impacts c-di-GMP levels and motility in response to peroxide stress. YfiN

contains a DGC domain that has been shown to mediate the synthesis of c-di-GMP (32, 33). We next assessed whether exposure to H₂O₂ impacted c-di-GMP levels. To measure c-di-GMP level, we used a *P_{cdrA}-gfp* promoter fusion on a plasmid to assess the transcriptional activity of *cdrA*, a gene that is positively regulated by c-di-GMP. We normalized the GFP signal to biomass using mKO (ex548/em559) inserted as a double copy at the *att* site on the chromosome and expressed constitutively. Bacteria were inoculated into glass-bottom dishes, grown as a monolayer of surface-attached cells, exposed to 1mM H₂O₂ (a concentration of peroxide that is well below the toxic levels analyzed above) and then imaged over the next 8h. Consistent with previous findings, the loss of YfiN reduced c-di-GMP levels, evident in the lower GFP signal (pink in images) for the $\Delta yfiN$ mutant compared to WT (**Fig 4A**, top left and bottom left, respectively, and **Fig. 4B**, time = 0). After exposure to peroxide, we observed an initial drop in GFP intensity by both WT and the $\Delta yfiN$ mutant (**Fig. 4B**), indicative of less c-di-GMP in response to hydrogen peroxide. Interestingly, while WT exhibited a recovery in c-di-GMP after ~6h, this recovery was not observed in the $\Delta yfiN$ mutant, for which the GFP signal remained low.

Figure 4. Loss of YfiN impacts c-di-GMP levels and motility in response to peroxide stress.

A) The WT strain and the $\Delta yfiN$ mutant carrying the P_{cdrA} -gfp fusion expressed from a multicopy plasmid were grown in glass bottom 8-well dishes containing M63 medium for 3h prior to the addition 1 mM H₂O₂, then imaged over 8h with a representative image from the 0h and 6h time points shown. The pink color shows GFP expression, and the blue color is contributed by the constitutively expressed mKO fluorescent protein. (B) Quantification of GFP signal intensity for the strains described in panel A. Fluorescent microscopy was used to determine GFP signal intensity as a measure of c-di-GMP production and normalized to the surface area of cells that constitutively expressed mKO fluorescent protein. Statistical significance was assessed with a Sidak's multiple comparisons post-test. (C) Representative images of the indicated strains grown on swarm assay plates 16h after inoculation, with and without 5 mM H₂O₂. (D) Quantification of plates described in panel C; all values are plotted after normalization to the WT not treated with H₂O₂, which was set to a value of 1. For panels B and D, error bars represent standard deviations of the results from three biological replicates each performed with three technical replicates. *, **, *** indicate differences that are significantly different at $P < 0.05$, $P < 0.01$, and $P < 0.0001$ respectively, compared to the WT. ns, non-significant.

Two key mechanisms whereby c-di-GMP regulates biofilm formation and maintenance are through (1) mediating increased production of extracellular polysaccharide (EPS) and/or (2) limiting motility. Previous findings from our lab did not observe a decrease in EPS production in the $\Delta yfiN$ mutant (31). To determine if the reduced biofilm phenotype in response to H_2O_2 observed for the $\Delta yfiN$ mutant was caused, at least in part, by increased motility in response to H_2O_2 , we first measured distance traveled by single cells imaged on glass well plates. Prior to the addition of 5mM H_2O_2 the motility of WT and the $\Delta yfiN$ mutant was comparable (**Fig. S3, Movies S1 and S2**). Thirty minutes after the addition of H_2O_2 , the motility of the $\Delta yfiN$ mutant was significantly higher than the WT (**Fig. S3, Movies S3 and S4**).

Figure S3. The $\Delta yfiN$ mutant exhibits significant increased motility in response to H_2O_2 . The WT strain and the $\Delta yfiN$ mutant were inoculated into glass well dishes for 1h. Motility was assessed over a 5 minute time course using brightfield microscopy prior to and 30 minutes after the addition of 5mM H_2O_2 . The TrackMate program was used to measure the tracks that each bacterium traveled over time, and the sum distance of all cell tracks was normalized to the number of bacteria at the start of imaging. Statistical significance was assessed via a Student's T-test. * indicates differences that are significantly different at $P < 0.05$, compared to the WT. ns, non-significant.

358 **Movie S1. Motility of WT prior to the addition of H₂O₂.** WT was inoculated into a glass well
 359 dish. Surface-attached bacteria were imaged one hour after inoculation with brightfield
 360 microscopy over a 5 minute time-course. The TrackMate program was used to identify bacteria
 361 (purple circles) and the distance of the tracks that bacteria traveled over time was measured and
 362 bacterial tracked distance depicted as a color-coded spectrum (blue<10μm; red>30μm).

378 **Movie S2. Motility of the $\Delta yfiN$ mutant prior to the addition of H₂O₂.** The $\Delta yfiN$ mutant was
 379 inoculated into a glass well dish and surface-attached bacteria were imaged 1h after inoculation,
 380 as described in Movie S1. The distance of the tracks that bacteria traveled over time was
 381 measured and bacterial tracked distance depicted as a color-coded spectrum (blue<10μm;
 382 red>30μm).

390

397 **Movie S3. Motility of WT after the addition of H₂O₂.** WT was inoculated into a glass well dish
 398 and allowed to attach to the surface for 1h. Bacteria were subsequently exposed to 5mM H₂O₂
 399 and imaged after 30 minutes for a 5 minute time-course using brightfield microscopy. The
 400 distance of the tracks that bacteria traveled over time was measured and bacterial tracked
 401 distance depicted as a color-coded spectrum (blue<10μm; red>30μm).
 402

417

418 **Movie S4. Motility of the $\Delta yfiN$ mutant after the addition of H₂O₂.** The $\Delta yfiN$ mutant was
 419 inoculated into a glass well dish and surface-attached bacteria were imaged 1h after inoculation
 420 as described in Movie S1. The distance of the tracks that bacteria traveled over time was
 421 measured and bacterial tracked distance depicted as a color-coded spectrum (blue<10μm;
 422 red>30μm).

We next assayed swarming on 0.5% agar plates in response to +/- 5mM H₂O₂. Swarming motility by *P. aeruginosa* is a surface-based motility which utilizes the flagellum. In the absence of H₂O₂, both strains exhibit comparable swarm motility (**Fig 4C-D**, left panels). While the addition of 5mM H₂O₂ resulted in almost complete suppression of swarming motility by the WT, the $\Delta yfiN$ mutant exposed to H₂O₂ still displayed swarming motility, although somewhat reduced from the no treatment control (**Fig 4C-D**, compare right panels to left panels). Collectively, these results suggest that *P. aeruginosa* uses YfiN to modulate c-di-GMP levels and suppress motility when exposed to peroxide stress.

Likely YfiBNR homologs are found in other bacterial genera. Known YfiN homologs are present in other biofilm-forming, Gram-negative bacteria like *Pseudomonas fluorescens*, *Escherichia coli*, and *Yersinia pestis* (36–39). Given the above evidence that YfiN regulates biofilm maintenance in *P. aeruginosa*, we mined GenBank for YfiBNR homologs in other bacteria, with a particular focus on Gram-negative, biofilm forming microbes. To identify potential homologs, we used the nucleotide sequence of the *P. aeruginosa* YfiBNR operon as a query in an NCBI Nucleotide BLAST search against the RefSeq representative genomes database (40). We aligned the 280 sequences with >75% query cover via Multiple Alignment using Fast Fourier Transform (MAFFT), then mapped sequences into a phylogenetic tree using FastTree 2.1.11 (41, 42).

The resulting phylogenetic tree (summarized in **Fig. 5A**) does not group by taxonomic classification and contains 116 species of bacteria in eight genera: 83.2%, 7.9%, and 3.9% of the hits were *Pseudomonas*, *Janithobacterium*, and *Serratia*, respectively. *Cupriavidus*, *Rugamonas*,

Inquilinus, *Bordetella*, and *Achromobacter* each made up 0.4-1.4% of the remaining sequences. The resulting phylogenetic tree identified a cluster (Fig. 5B) with high bootstrap support that formed around the query sequence. This cluster contains *Pseudomonas* and *Serratia*, which are genera of different orders. Notably, the cluster includes a sequence from *Serratia marcescens*, a known biofilm-forming, opportunistic human pathogen that infects the respiratory tract, urinary tract and eyes (43–45). These YfiBNR homologs may be similarly involved in biofilm maintenance and response to peroxide stress in their respective species.

Figure 5: YfiBNR homologs are found in multiple bacterial genera. A) Potential YfiBNR homologs were identified via a Nucleotide BLAST search, aligned with MAFFT, arranged into a phylogenetic tree using FastTree 2.1.11, and visualized using Interactive Tree of Life software (46). Sequences most closely mapped to sequences of the same genus were collapsed to generate the above abbreviated tree. The full tree is shown in Figure S4. Each branch is labeled with the number of sequences from that genus present on that branch in the full tree. The branch containing the *P. aeruginosa* YfiBNR query sequence is marked in red. Branch lengths are not mapped on this tree. B) A cluster labeled with high bootstrap values, indicating frequent mapping of the same branches in various iterations of the tree, formed around the YfiBNR query sequence, which is marked in red. Branch lengths, which mark how much genetic divergence exists between sequences, are to scale. The sequences mapped most closely to YfiBNR were

from multiple other *Pseudomonas* and *Serratia* strains, suggesting potential homology across species and genera.

Figure S4. Full phylogenetic tree of potential YfiBNR homologs. The full phylogenetic tree of potential YfiBNR homologs was produced from 280 aligned sequences from 116 species in eight genera.

Discussion

YfiN homologs are found among various bacterial genera, including *Pseudomonas*, *Escherichia*, *Salmonella* and *Serratia*, and this protein has been shown to play a role in regulating intracellular c-di-GMP in *P. aeruginosa* here and in previous studies (32, 47). The contributions of YfiN have been largely studied in the context of motility and biofilm formation, whereby a loss of YfiN leads to less c-di-GMP and reduced biofilm formation (32, 48), consistent with our results in *P. aeruginosa*. Increased motility and the resulting dispersal of the biofilm that we observe here has been indirectly supported through the study of upstream regulators. For instance, deletion of YfiR, an upstream inhibitor of YfiN, has been shown to repress motility in *P. aeruginosa* PAO1 (33). A release of inhibition by YfiR leads to activation of the c-di-GMP-producer YfiN, thus reducing motility. Furthermore, previous biochemical studies have shown that oxidized glutathione disrupts disulfide bond formation and protein stability of YfiR (35), suggesting the critical role of redox stress on the regulation of this pathway.

Here we observe a significant biofilm maintenance defect at 48h, but not 12h, in the $\Delta yfiN$ mutant. Consistent with the loss of this DGC, we observe a significant decrease in *cdrA* transcription, an indirect read-out of c-di-GMP level, in the $\Delta yfiN$ mutant compared to WT. We also note increased cell death and a modest increase in dispersion in the $\Delta yfiN$ mutant compared to WT. Thus, we attribute this loss of biofilm maintenance both to enhanced dispersal and increased cell death. We provide evidence that YfiN modulates c-di-GMP levels, cellular motility and the ability to maintain the biofilm in response to peroxide (**Fig. 6**). At this stage, we have not established the link between the loss of YfiN and the observed cell death and dispersion phenotypes. Low levels of c-di-GMP are associated with increased surface motility of *P.*

495 *aeruginosa*, with our group and many others contributing to the known mechanisms (12, 18, 49,
 496 50). We presume that c-di-GMP-mediated regulation of motility is through one of the known
 497 mechanisms, but this remains to be demonstrated. Why we observe increased cell death in the
 498 *yfiN* mutant treated with peroxide is a mystery. Our previous work strongly suggested that loss
 499 of RmcA/MorA contributed to cell death during starvation because strains lacking these proteins
 500 continued to use resources to make EPS even when starving; the mutants were likely starving
 501 themselves to death (30). Here, we suggest two possibilities for the cell death observation. The
 502 first is that high c-di-GMP levels promoted by YfiN are necessary to induce protective responses
 503 to peroxide. Increased c-di-GMP in *Vibrio cholerae* has been found to induce matrix production
 504 and enhance catalase activity (51, 52). In further support of this view in *P. aeruginosa*, the DGC
 505 PA3177 has been found to synthesize c-di-GMP in response to oxidative hypochlorite (53) and
 506 to mediate H₂O₂ and antibiotic tolerance (54). Furthermore, sublethal concentrations of H₂O₂
 507 have been shown to select for mutations in the gene encoding for WspF, which represses the
 508 DGC WspR (55). The second possibility is that exposure to peroxide in the absence of YfiN
 509 results in the biofilm bacteria returning to a less tolerant, planktonic lifestyle. This second
 510 hypothesis is supported by the observation that YfiN mutants are more motile and more readily
 511 dispersed from the biofilm subsequent to peroxide exposure.

512

513 **Figure 6: Model for *P. aeruginosa* YfiN's contribution to biofilm maintenance in response to**
514 **peroxide exposure.** YfiN is not a major contributor to early biofilm formation in our growth
515 conditions (top panel). In a mature WT biofilm (bottom panel), YfiN is needed to regulate
516 maintenance through the modulation of c-di-GMP and motility in response to peroxide. The loss
517 of YfiN results in biofilms that are maintenance-deficient and exhibit increased cell death and
518 dispersal upon exposure to exogenous peroxide, and perhaps in response to endogenous redox
519 active molecules.

520

521 Interestingly, we found that loss of biofilm biomass in later-stage biofilms in strains
522 lacking YfiN function in both the absence and presence of H₂O₂, and furthermore, the loss of
523 biofilm biomass by the $\Delta yfiN$ mutant in the absence of H₂O₂ could be rescued by replenishing
524 biofilms with fresh medium. These data suggest that YfiN may be important for adapting to
525 oxidative stressors generally present in the environmental milieu, including self-produced redox
526 active compounds produced by *P. aeruginosa*. Phenazines, small redox active molecules that can
527 act as alternate electron acceptors, may be one such molecule. Interestingly, phenazines have
528 been linked to EPS production via the DGC RmcA (56, 57) and we have shown that RmcA is
529 critical for biofilm maintenance in response to starvation (30), thus there are multiple lines of
530 evidence linking phenazines to the control of biofilm biology. Further work is needed to
531 understand if the ability for YfiN to regulate biofilm maintenance is specific to peroxide

exposure or if YfiN is more broadly required across a range of redox active agents, including phenazines and immune system-generated molecules with similar chemistry (58–62).

Overall, our data support the model we proposed previously (30), whereby *P. aeruginosa* actively maintains a mature biofilm even in the face of environmental stressors. That is, *P. aeruginosa* appears to utilize at least three different c-di-GMP metabolizing enzymes, RmcA, MorA and YfiN, to actively maintain the biofilm in response to environmental perturbations, including starvation or peroxide exposure. Based on work here and our previous study of RmcA and MorA, the loss of the ability to modulate c-di-GMP in a mature biofilm in the face of environmental stressors has dire consequences for the cell, that is, death. If one also considers that published work showing that *P. aeruginosa* has pathways for the generation of “maintenance energy” in the context of mature biofilms (63–65), it appears that this microbe has a robust set of mechanisms to sense and respond to environmental inputs when in a biofilm, and then regulate its response(s) appropriately. Such biofilm maintenance pathways might be key to preventing biofilm bacteria from “over-reacting” to transient stresses; thus mediating persistence through the perturbation without transitioning to a planktonic state. That is, perhaps biofilms have a type of “fight” or “flight” response regulated by environmental inputs and c-di-GMP levels. We speculate that such “biofilm maintenance” pathways are likely present in other microbes, and furthermore, at least a subset of c-di-GMP-related enzymes for which there is no assigned function play a role in such pathways.

A number of microbes have been shown to have the *yfi* operon, which encodes the tripartite regulatory system of YfiN, YfiR and YfiB. The current model suggests that YfiR is localized to the periplasm, where it directly inhibits YfiN. This inhibition is relieved in the presence of oxidative stress, which leads to misfolding of YfiR. Additionally, YfiB potentially

sequesters YfiR following cell envelope stress, thus relieving inhibition of YfiN (47). Therefore, it is likely that both oxidative and cell envelope stress can regulate YfiN activity; our biofilm and motility results support the previously reported effects of oxidative stress (66). Moreover, YfiN has been reported to localize to the mid-cell and arrest division in *Salmonella enterica* and *Escherichia coli* and regulate cell division in response to reductive stress (67). Interestingly, increased mid-cell localization was not observed in *P. aeruginosa* (67), indicating that this protein contributes to different functions in different organisms.

The YfiBNR system shows conservation among other Gram-negative microbes beyond *E. coli*, including those organisms known to make biofilms. *Serratia* and *Yersinia*, also in the family Yersiniaceae, utilize the homologous system HmsCDE (38). Like YfiBNR, the HmsCDE system is implicated in biofilm growth and response to redox stress. Deletion of the *yfiN* homolog, *hmsD*, in *Y. pestis* leads to decreases for *in vitro* and *in vivo* biofilm formation (68). Null mutants of *hmsD* exhibit decreased biofilm biomass in reducing environments (69); these previous findings and our work here are consistent with the importance of YfiN in biofilm maintenance, particularly in response to redox conditions. Furthermore, in liquid culture, HmsD and HmsC (YfiR homolog) expression is more than doubled during the late stationary phase, and compared to the control, the $\Delta hmsD$ mutant exhibits a severe growth defect when grown in media containing H₂O₂. We did not observe increased sensitivity of the $\Delta yfiN$ mutant to H₂O₂ in planktonic conditions, again emphasizing the differential functions of this system in different organisms.

MATERIALS AND METHODS

Strains and media. UCBPP-PA14 (PA14) was used in all the experiments. *P. aeruginosa* was routinely streaked onto lysogeny broth (LB) plates containing 1.5% agar prior to overnight culturing in LB liquid cultures at 37°C. As needed, LB was supplemented with 10mg/ml gentamicin (Gm) and 50mg/ml kanamycin (Kan). Biofilm medium used in static 96-well crystal violet assays was composed of M63 medium supplemented with 1 mM MgSO₄ and 0.4% (wt/vol) L-arginine monochloride. For medium replacement assays, plates were grown as indicated, spent medium discarded and biofilms washed twice prior to the addition of fresh medium. To limit the formation bubbles caused by the addition of H₂O₂ during microfluidic-based biofilm assays, we used buffered KA biofilm medium, a modification K10T medium that contains 50mM Tris-HCl (pH 7.4), 0.61mM MgSO₄, and 0.4% arginine (70).

Static biofilm assays and quantification. Overnight cultures were inoculated into 96-well U-bottom polystyrene plates (Costar) containing M63-based biofilm medium and grown for the specified time at 37°C in a hydrated container prior to washing, staining with crystal violet (CV) and solubilization of the CV stain with 30% glacial acetic acid (71). The presence of the biofilm was quantified by measuring the extent of biofilm-associated CV measured in a spectrophotometer to determine the optical density at 500 nm (OD₅₅₀).

For biofilms imaged microscopically in static assays, overnight cultures were prepared as described above and inoculated into 8-well glass-bottom dishes containing M63 biofilm medium and grown at 37°C for the desired length of time prior to imaging with a Nikon Eclipse Ti inverted microscope where a minimum of 6 fields of view were captured. To assess the viability

of biofilms grown in glass bottom dishes, growth medium was discarded, biofilms washed twice with fresh medium, and the biofilms immersed in fresh medium containing propidium iodide 10 min prior to imaging. To assess the concentration of c-di-GMP we utilized P_{cdrA} -*gfp* fusion expressed from a multicopy pMQ72 plasmid (72) which was maintained in overnight cultures supplemented with Gm prior to inoculation into antibiotic-free M63 biofilm medium.

Microfluidics. Biofilms were visualized under flow in microfluidics chambers kindly provided by the Nadell laboratory at Dartmouth College. Chambers used polydimethylsiloxane (PDMS) bonded to a cover glass (1.5 × 36 mm × 60 mm; Thermo Fisher, Waltham MA) through soft lithography techniques (73, 74). Overnight bacterial cultures were centrifuged, resuspended in biofilm medium, adjusted to an OD₆₀₀ of 1, pipetted into microfluidics chambers, and allowed to attach for 1 h. Tubing (catalog no. 30 Cole Palmer PTFE) to transport influent and effluent medium was attached first to BD 5-ml syringes containing biofilm medium, then to the microfluidics chambers, and then to syringe pumps (Pico Plus Elite; Harvard Apparatus) operating at a flow rate of 0.75 μl/min.

Image acquisition and data analysis. All microscope images were acquired using Nikon Elements AR driving a Nikon Eclipse Ti inverted microscope and a Hamamatsu ORCA-Flash 4.0 camera. Samples were imaged with either a Plan Apochromat 100 DM Oil or Plan Fluor 40 DIC M N2 objective. Fast scan mode and 2X2 binning were used for imaging. All images were collected in a temperature controlled environmental chamber set to 37°C. Images were processed with background subtraction and signal strength quantified by measuring the mean signal intensity/pixel through the Integrated Density (IntDen) function. To quantify single-cell motility, images were inverted, a threshold applied and the TrackMate program (75) used.

Statistical analysis. Data were analyzed using GraphPad Prism 8. Unless otherwise noted, data are representative of the results from at least three independent experiments. A Student T-test with a Dunnett's or Sidak's multiple comparisons post-test, as indicated, were used to compare results and to assess significance.

Potential homolog identification and phylogenetic tree construction. The nucleotide sequence of YfiBNR in PAO1 (NC_002516.2:1212717-1215074) was used as a query in a Nucleotide BLAST search (40). Bacterial genomes within the RefSeq Representative genomes database were searched with discontinuous megablast. The expectant threshold was set to 0.0001; all other default algorithm parameters were used. Sequences with >75% query cover were aligned using MAFFT (41). FastTree 2.1.11 was used to construct a phylogenetic tree based on the aligned sequences, using the GTR+CAT model and Gamma20-based likelihoods (42). 1000 re-samples were used to calculate bootstrap support for the tree, and a nonhomologous human protein was used as an outgroup to root the tree. Interactive Tree of Life version 4 was used to visualize the phylogenetic tree.

Acknowledgements. We thank Carey Nadell for providing microfluidic devices, Zdenek Svindrych for providing imaging guidance, Olga Zhaxybayeva for providing bioinformatic advice, and Thomas Wood for generously and speedily sending strains. We also thank Gillian Kenyon for transformation selected strains with fluorescent marker plasmids. This work was supported by the NIH (R37 AI83256 to GAO) and the James O. Freedman Presidential Scholar Award to SAK.

Literature Cited.

1. Khan NH, Ishii Y, Kimata-Kino N, Esaki H, Nishino T, Nishimura M, Kogure K. 2007. Isolation of *Pseudomonas aeruginosa* from open ocean and comparison with freshwater, clinical, and animal isolates. *Microb Ecol* 53:173–186.
2. Donlan RM. 2001. Biofilms and device-associated infections. *Emerg Infect Dis* 7:277–281.
3. Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. 2016. Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. *BMC Pulm Med* 16:1–22.
4. Van Acker H, Van Dijck P, Coenye T. 2014. Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. *Trends Microbiol* 22:326–333.
5. Stewart PS, Franklin MJ, Williamson KS, Folsom JP, Boegli L, James GA. 2015. Contribution of stress responses to antibiotic tolerance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 59:3838–3847.
6. Armbruster CR, Lee CK, Parker-Gilham J, de Anda J, Xia A, Tseng BS, Hoffman LR, Jin F, Harwood CS, Wong GCL, Parsek MR. 2019. Heterogeneity in surface sensing produces a division of labor in *Pseudomonas aeruginosa* populations. *Elife* 8:1–29.
7. Utada AS, Bennett RR, Fong JCN, Gibiansky ML, Yildiz FH, Golestanian R, Wong GCL. 2014. *Vibrio cholerae* use pili and flagella synergistically to effect motility switching and conditional surface attachment. *Nat Commun* 5:1–8.
8. Luo Y, Zhao K, Baker AE, Kuchma SL, Coggan KA, Wolfgang MC, Wong GCL, O’Toole GA. 2015. A hierarchical cascade of second messengers regulates *Pseudomonas aeruginosa* surface behaviors. *MBio* 6:1–11.
9. Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GCL, Parsek MR.

2011. The Pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. PLoS Pathog 7: e1001264.
10. Toutain CM, Caizza NC, Zegans ME, O'Toole GA. 2007. Roles for flagellar stators in biofilm formation by *Pseudomonas aeruginosa*. Res Microbiol 158:471–477.
11. Conrad JC, Gibiansky ML, Jin F, Gordon VD, Motto DA, Mathewson MA, Stopka WG, Zelasko DC, Shrout JD, Wong GCL. 2011. Flagella and pili-mediated near-surface single-cell motility mechanisms in *P. aeruginosa*. Biophys J 100:1608–1616.
12. Kuchma SL, Ballok AE, Merritt JH, Hammond JH, Lu W, Rabinowitz JD, O'Toole GA. 2010. Cyclic-di-GMP-mediated repression of swarming motility by *Pseudomonas aeruginosa*: The *pilY1* gene and its impact on surface-associated behaviors. J Bacteriol 192:2950–2964.
13. Kulesekara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S, Lee DG, Neely AN, Hyodo M, Hayakawa Y, Ausubel FM, Lory S. 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proc Natl Acad Sci U S A 103:2839–2844.
14. Hickman JW, Tifrea DF, Harwood CS. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A 102:14422–14427.
15. Baraquet C, Murakami K, Parsek MR, Harwood CS. 2012. The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the *pel* operon promoter in response to c-di-GMP. Nucleic Acids Res 40:7207–7218.
16. Friedman L, Kolter R. 2004. Genes involved in matrix formation in *Pseudomonas*

aeruginosa PA14 biofilms. Mol Microbiol 51:675–690.

17. Paul R, Weiser S, Amiot NC, Chan C, Schirmer T, Giese B, Jenal U. 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes Dev 18:715–727.
18. Simm R, Morr M, Kader A, Nimtz M, Römling U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol Microbiol 53:1123–1134.
19. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol 7:263–273.
20. Danese PN, Pratt LA, Kolter R. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J Bacteriol 182:3593–3596.
21. Ghafoor A, Hay ID, Rehm BHA. 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. Appl Environ Microbiol 77:5238–5246.
22. Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S. 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. Proc Natl Acad Sci U S A 102:16819–16824.
23. Flemming HC, Wingender J. 2001. Relevance of microbial extracellular polymeric substances (EPSs) - Part I: Structural and ecological aspects. Water Sci Technol 43:1–8.
24. Yan J, Nadell CD, Stone HA, Wingreen NS, Bassler BL. 2017. Extracellular-matrix-mediated osmotic pressure drives *Vibrio cholerae* biofilm expansion and cheater exclusion. Nat Commun 8:1–11.
25. Jang IA, Kim J, Park W. 2016. Endogenous hydrogen peroxide increases biofilm formation by inducing exopolysaccharide production in *Acinetobacter oleivorans* DR1.

Sci Rep 6:1–12.

26. Zhao Y, Cai Y, Chen Z, Li H, Xu Z, Li W, Jia J, Sun Y. 2021. SpoT-Mediated NapA upregulation promotes oxidative stress-induced *Helicobacter pylori* biofilm formation and confers multidrug resistance. *Antimicrob Agents Chemother* 65:e00152-21.
27. Wei Q, Le Minh PN, Dötsch A, Hildebrand F, Panmanee W, Elfarash A, Schulz S, Plaisance S, Charlier D, Hassett D, Häussler S, Cornelis P. 2012. Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nucleic Acids Res* 40:4320–4333.
28. Hassett DJ, Cohen MS. 1989. Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *FASEB J* 3:2574–2582.
29. Lau GW, Britigan BE, Hassett DJ. 2005. *Pseudomonas aeruginosa* OxyR is required for full virulence in rodent and insect models of infection and for resistance to human neutrophils. *Infect Immun* 73:2550–2553.
30. Katharios-Lanwermeier S, Whitfield GB, Howell PL, O'toole GA. 2021. *Pseudomonas aeruginosa* uses c-di-GMP phosphodiesterases RmcA and MorA to regulate biofilm maintenance. *MBio* 12: e03384-20.
31. Ha DG, Richman ME, O'Toole GA. 2014. Deletion mutant library for investigation of functional outputs of cyclic diguanylate metabolism in *Pseudomonas aeruginosa* PA14. *Appl Environ Microbiol* 80:3384–3393.
32. Ueda A, Wood TK. 2009. Connecting quorum sensing, c-di-GMP, Pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog* 5:1–15.
33. Malone JG, Jaeger T, Spangler C, Ritz D, Spang A, Arrieumerlou C, Kaeffer V,

- Landmann R, Jenal U. 2010. YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. PLoS Pathog 6:1–17.
34. Malone JG, Jaeger T, Manfredi P, Dötsch A, Blanka A, Bos R, Cornelis GR, Häussler S, Jenal U. 2012. The YfiBNR signal transduction mechanism reveals novel targets for the evolution of persistent *Pseudomonas aeruginosa* in cystic fibrosis airways. PLoS Pathog 8:1–19.
35. Yang X, Yang XA, Xu M, Zhou L, Fan Z, Jiang T. 2015. Crystal structures of YfiR from *Pseudomonas aeruginosa* in two redox states. Biochem Biophys Res Commun 461:14–20.
36. Giddens SR, Jackson RW, Moon CD, Jacobs MA, Zhang X-X, Gehrig SM, Rainey PB. 2007. Mutational activation of niche-specific genes provides insight into regulatory networks and bacterial function in a complex environment. Proc Natl Acad Sci 104:18247–18252.
37. Sanchez-Torres V, Hu H, Wood TK. 2010. GGDEF proteins YeaI, YedQ, and YfiN reduce early biofilm formation and swimming motility in *Escherichia coli*. Appl Microbiol Biotechnol 2010 90:651–658.
38. Ren GX, Yan HQ, Zhu H, Guo XP, Sun YC. 2014. HmsC, a periplasmic protein, controls biofilm formation via repression of HmsD, a diguanylate cyclase in *Yersinia pestis*. Environ Microbiol 16:1202–1216.
39. Hufnagel DA, DePas WH, Chapman MR. 2014. The disulfide bonding system suppresses CsgD-Independent cellulose production in *Escherichia coli*. J Bacteriol 196:3690–3699.
40. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs.

Nucleic Acids Res 25: 3389-3402.

41. Katoh K, Rozewicki J, Yamada KD. 2018. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform 20:1160–1166.
42. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2 - Approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490.
43. Maki DG, Hennekens CG, Phillips CW, Shaw W V., Bennett J V. 1973. Nosocomial urinary tract infection with *Serratia marcescens*: An epidemiologic study. J Infect Dis 128:579–587.
44. yu VL. 1979. *Serratia marcescens*: Historical perspective and clinical review. N Engl J Med 300:887–893.
45. Kalivoda EJ, Stella NA, O’Dee DM, Nau GJ, Shanks RMQ. 2008. The cyclic AMP-dependent catabolite repression system of *Serratia marcescens* mediates biofilm formation through regulation of type 1 fimbriae. Appl Environ Microbiol 74:3461–3470.
46. Letunic I, Bork P. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res 47:W256–W259.
47. Giardina G, Paiardini A, Fernicola S, Franceschini S, Rinaldo S, Stelitano V, Cutruzzolà F. 2013. Investigating the allosteric regulation of YfiN from *Pseudomonas aeruginosa*: Clues from the structure of the catalytic domain. PLoS One 8:1–15.
48. Le Guyon S, Simm R, Rehn M, Römling U. 2015. Dissecting the cyclic diguanylate monophosphate signalling network regulating motility in *Salmonella enterica* serovar Typhimurium. Environ Microbiol 17:1310–1320.
49. Wolfe AJ, Visick KL. 2008. Get the message out: Cyclic-di-GMP regulates multiple levels of flagellum-based motility. J Bacteriol 190:463–475.

50. Kuchma SL, Brothers KM, Merritt JH, Liberati NT, Ausubel FM, O'Toole GA. 2007. BifA, a cyclic-di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J Bacteriol* 189:8165–8178.
51. Krasteva P V., Fong JCN, Shikuma NJ, Beyhan S, Navarro MVAS, Yildiz FH, Sondermann H. 2010. *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. *Science* 327:866–868.
52. Fernandez NL, Waters CM. 2019. Cyclic di-GMP increases catalase production and hydrogen peroxide tolerance in *Vibrio cholerae*. *Appl Environ Microbiol* 85:e01043-19.
53. Stempel N, Nusser M, Neidig A, Brenner-Weiss G, Overhage J. 2017. The oxidative stress agent hypochlorite stimulates c-di-GMP Synthesis and biofilm formation in *Pseudomonas aeruginosa*. *Front Microbiol* 0:2311.
54. Poudyal B, Sauer K. 2018. The PA3177 gene encodes an active diguanylate cyclase that contributes to biofilm antimicrobial tolerance but not biofilm formation by *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 62: e01049-18.
55. Chua SL, Ding Y, Liu Y, Cai Z, Zhou J, Swarup S, Drautz-Moses DI, Schuster SC, Kjelleberg S, Givskov M, Yang L. 2016. Reactive oxygen species drive evolution of pro-biofilm variants in pathogens by modulating cyclic-di-GMP levels. *Open Biol* 6:160162.
56. Dietrich LEP, Okegbe C, Price-Whelan A, Sakhtah H, Hunter RC, Newmana DK. 2013. Bacterial community morphogenesis is intimately linked to the intracellular redox state. *J Bacteriol* 195:1371–1380.
57. Okegbe C, Fields BL, Cole SJ, Beierschmitt C, Morgan CJ, Price-Whelan A, Stewart RC, Lee VT, Dietrich LEP. 2017. Electron-shuttling antibiotics structure bacterial communities by modulating cellular levels of c-di-GMP. *Proc Natl Acad Sci U S A*

806 114:E5236–E5245.

807 58. Panmanee W, Hassett DJ. 2009. Differential roles of OxyR-controlled antioxidant
808 enzymes alkyl hydroperoxide reductase (AhpCF) and catalase (KatB) in the protection of
809 *Pseudomonas aeruginosa* against hydrogen peroxide in biofilm vs. planktonic culture.
810 FEMS Microbiol Lett 295:238–244.

811 59. Thomson E, Brennan S, Senthilmohan R, Gangell CL, Chapman AL, Sly PD, Kettle AJ.
812 2010. Identifying peroxidases and their oxidants in the early pathology of cystic fibrosis.
813 Free Radic Biol Med 49:1354–1360.

814 60. Galli F, Battistoni A, Gambari R, Pompella A, Bragonzi A, Pilolli F, Iuliano L, Piroddi M,
815 Dehecchi MC, Cabrini G. 2012. Oxidative stress and antioxidant therapy in cystic
816 fibrosis. Biochim Biophys Acta - Mol Basis Dis 1822:690–713.

817 61. Robinson JM. 2008. Reactive oxygen species in phagocytic leukocytes. Histochem Cell
818 Biol 2008 130:281–297.

819 62. Fang FC. 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and
820 controversies. Nat Rev Microbiol 2004 2:820–832.

821 63. Goodwine J, Gil J, Doiron A, Valdes J, Solis M, Higa A, Davis S, Sauer K. 2019.
822 Pyruvate-depleting conditions induce biofilm dispersion and enhance the efficacy of
823 antibiotics in killing biofilms in vitro and in vivo. Sci Rep 9:1–16.

824 64. Lin YC, Cornell WC, Jo J, Price-Whelan A, Dietrich LEP. 2018. The *Pseudomonas*
825 *aeruginosa* complement of lactate dehydrogenases enables use of d- and l-lactate and
826 metabolic cross-feeding. MBio 9:1–12.

827 65. Basta DW, Bergkessel M, Newman DK. 2017. Identification of fitness determinants
828 during energy-limited growth arrest in *Pseudomonas aeruginosa*. MBio 8: e01170-17.

- 829 66. Varghese A, Ray S, Verma T, Nandi D. 2020. Multicellular string-like structure formation
830 by *Salmonella typhimurium* depends on cellulose production: Roles of diguanylate
831 cyclases, YedQ and YfiN. Front Microbiol 11:1–15.
- 832 67. Kim HK, Harshey RM. 2016. A diguanylate cyclase acts as a cell division inhibitor in a
833 two-step response to reductive and envelope stresses. MBio 7:e00822-16.
- 834 68. Sun YC, Koumoutsi A, Jarrett C, Lawrence K, Gherardini FC, Darby C, Hinnebusch BJ.
835 2011. Differential control of *Yersinia pestis* biofilm formation in vitro and in the flea
836 vector by two c-di-gmp diguanylate cyclases. PLoS One 6: e19267.
- 837 69. Ren GX, Fan S, Guo XP, Chen S, Sun YC. 2016. Differential regulation of c-di-GMP
838 metabolic enzymes by environmental signals modulates biofilm formation in *Yersinia*
839 *pestis*. Front Microbiol 7:1–12.
- 840 70. Collins AJ, Pastora AB, Jarrod Smith T, O'Toole GA. 2020. MapA, a second large RTX
841 adhesin conserved across the Pseudomonads, contributes to biofilm formation by
842 *Pseudomonas fluorescens*. J Bacteriol: e00277-20.
- 843 71. O'Toole GA, Kolter R. 1998. Flagellar and twitching motility are necessary for
844 *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295–304.
- 845 72. Shanks RMQ, Caiazza NC, Hinsa SM, Toutain CM, O'Toole GA. 2006. *Saccharomyces*
846 *cerevisiae*-based molecular tool kit for manipulation of genes from Gram-negative
847 bacteria. Appl Environ Microbiol 72:5027–5036.
- 848 73. Sia SK, Whitesides GM. 2003. Microfluidic devices fabricated in poly(dimethylsiloxane)
849 for biological studies. Electrophoresis 24:3563–3576.
- 850 74. Weibel DB, DiLuzio WR, Whitesides GM. 2007. Microfabrication meets microbiology.
851 Nat Rev Microbiol 5:209–218.

852 75. Tinevez JY, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, Bednarek
853 SY, Shorte SL, Eliceiri KW. 2017. TrackMate: An open and extensible platform for
854 single-particle tracking. *Methods* 115:80–90.
855