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Nitric Oxide-Mediated Induction of Dispersal in *Pseudomonas aeruginosa* Biofilms Is Inhibited by Flavohemoglobin Production and Is Enhanced by Imidazole

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ABSTRACT The biological signal molecule nitric oxide (NO) was found to induce biofilm dispersal across a range of bacterial species, which led to its consideration for therapeutic strategies to treat biofilms and biofilm-related infections. However, biofilms are often not completely dispersed after exposure to NO. To better understand this phenomenon, we investigated the response of *Pseudomonas aeruginosa* biofilm cells to successive NO treatments. When biofilms were first pretreated with a low, noneffective dose of NO, a second dose of the signal molecule at a concentration usually capable of inducing dispersal did not have any effect. Amperometric analysis revealed that pretreated *P. aeruginosa* cells had enhanced NO-scavenging activity, and this effect was associated with the production of the flavohemoglobin Fhp. Further, quantitative real-time reverse transcription-PCR (qRT-PCR) analysis showed that *fhp* expression increased by over 100-fold in NO-pretreated biofilms compared to untreated biofilms. Biofilms of mutant strains harboring mutations in *fhp* or *fhpR*, encoding a NO-responsive regulator of *fhp*, were not affected in their dispersal response after the initial pretreatment with NO. Overall, these results suggest that FhpR can sense NO to trigger production of the flavohemoglobin Fhp and inhibit subsequent dispersal responses to NO. Finally, the addition of imidazole, which can inhibit the NO dioxygenase activity of flavohemoglobin, attenuated the prevention of dispersal after NO pretreatment and improved the dispersal response in older, starved biofilms. This study clarifies the underlying mechanisms of impaired dispersal induced by repeated NO treatments and offers a new perspective for improving the use of NO in biofilm control strategies.

KEYWORDS *Pseudomonas aeruginosa*, biofilms, dispersal, flavohemoglobin, nitric oxide

The biofilm mode of growth, in which microorganisms form aggregates that are encased in a matrix of extracellular macromolecules and can be either suspended or attached to a surface, is essential to the ecology and biology of bacteria (1, 2). Cells in biofilms are generally characterized by a high level of genetic and physiological heterogeneity. This heterogeneity of biofilms, which is caused by microscale chemical gradients, adaptation to environmental conditions, stochastic gene expression, and genotypic variation (3), is central to the function of biofilms and often leads to increased resistance to external stressors, biocides, antibiotics, and immune systems compared to free-floating planktonic cells (4, 5). The increased resistance of biofilms to antimicrobials, which can lead to the failure of treatment strategies to control them, is

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the cause of many problems in industrial and clinical settings. In the food industry, biofilms limit the run length of manufacturing plants and cause product spoilage and safety problems (6). In drinking water distribution systems, biofilms can cause corrosion or increase the risk of releasing microbial pathogens into the water (7). In clinical settings, formation of a biofilm by the opportunistic pathogen *Pseudomonas aeruginosa* is responsible for many infections, including chronic lung infections in cystic fibrosis patients and wound infection in severe-burn victims (8). Hence, it is of great interest to study biofilms with the aim of inhibiting biofilm formation.

Bacterial biofilms develop through several stages, including initial reversible attachment, irreversible attachment, maturation, and dispersal (9). The final stage, dispersal, is an active, highly regulated response of biofilm bacteria that is different from the passive, mechanically induced sloughing and erosion of cells from the biofilm outer layers (10, 11). Biofilm dispersal can be triggered by a variety of environmental cues, including changes in nutrient availability and iron level (12–15), changes in temperature (16), and oxygen depletion (17, 18), as well as low levels of nitric oxide (NO) (11, 19). NO, which is a hydrophobic, highly reactive, and short-lived free radical that can diffuse freely through cellular membranes, is a widespread biological messenger. Previous research has found that low, nontoxic concentrations (in the nanomolar to low micromolar range) of NO cause a transition from the sessile biofilm to the motile planktonic phenotype in bacteria. The role of NO in mediating biofilm dispersal was first identified in *P. aeruginosa* (19) and was later found to be conserved across a broad range of microorganisms (references 20, 21, 22, and 23; reviewed in reference 24), as well as multispecies biofilms from water systems (25–28). Overall, these observations suggested a promising approach to define novel strategies based on NO delivery to disperse and control biofilms and biofilm-associated infections with applications in both clinical and industrial contexts (29).

Before the identification of NO as a signal for biofilm dispersal, the role of NO in bacteria had been mostly studied in the context of nitrosative stress, when NO is produced at higher concentrations from endogenous sources in bacteria or by immune response defenses, such as macrophages (30). These studies have led to the characterization of a number of cellular systems capable of responding to NO to alleviate nitrosative stress. Upon reaction with oxygen or superoxide, NO generates reactive nitrogen species (RNS) that can broadly damage cellular proteins, for instance, by attacking cysteine thiols or iron-sulfur (Fe-S) clusters in respiratory and other metabolic enzymes, as well as nucleic acids and lipids (31). To prevent such damage, bacteria possess a number of NO-sensing proteins, which contain redox sensors, such as heme iron cofactors, non-heme iron or copper centers, Fe-S clusters, or cysteine thiols, and can regulate specific defense responses (32–35). For example, *P. aeruginosa* can detoxify NO by using either a NO reductase (NOR) (36) or a flavohemoglobin, Fhp (36, 37). Fhp activity requires oxygen, and the system is thus used under aerobic conditions, while NOR is induced under anaerobic conditions. In *P. aeruginosa*, induction of NOR occurs via the heme NO sensor-containing transcription regulator DNR (dissimilatory nitrate respiration regulator) (38), while Fhp is regulated by FhpR (37), which is homologous to the non-heme iron cofactor protein NorR, which is capable of sensing NO in *Escherichia coli* (39). The NO-sensing and protective mechanisms appear to be highly diverse in bacteria, and in *E. coli*, NorR regulates a flavorubredoxin, NorV, rather than the Fhp homologue flavohemoglobin Hmp (40), the expression of which is regulated by the Fe-S cluster regulator NsrR (41). In *E. coli* and *P. aeruginosa*, the interplay between NO-producing and NO-scavenging systems was recently shown to generate oscillating cycles of NO levels, thus ensuring that intracellular NO remains present at nontoxic levels (42, 43). In several bacterial species, the molecular mechanisms of NO-mediated dispersal have been shown to involve bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP), a soluble second messenger, which is the key element of a genetic network governing motile-to-sessile transitions and is conserved in Gram-negative bacteria. In *P. aeruginosa*, although a direct sensor of NO that mediates c-di-GMP degradation has yet to be identified, recent studies have uncovered a novel NO-sensing

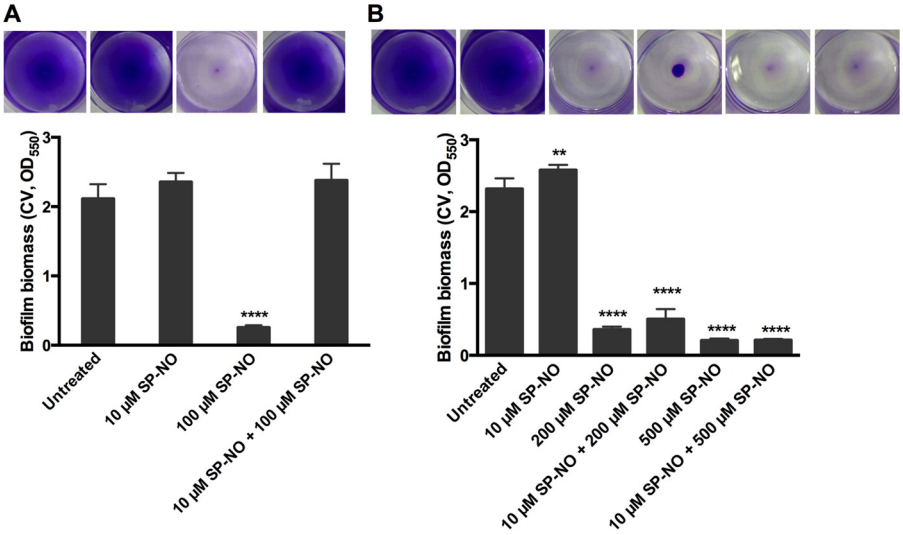

FIG 1 Biofilms that were pretreated with a low dose of NO required increased concentrations of NO to induce dispersal. *P. aeruginosa* biofilms were grown in multiwell plate batch cultures for a total of 6 h 15 min, including or not a pretreatment with 10 μM SP-NO at $t = 3$ h and a dispersal treatment with 100 μM (A), 200 μM (B), or 500 μM (B) SP-NO at $t = 6$ h. At the end of the incubation, biofilm biomass was analyzed by CV staining. Experimental details are shown in Fig. S1A in the supplemental material. The error bars indicate standard deviations ($n = 3$). The asterisks indicate statistically significant differences compared to untreated control samples (**, $P < 0.01$; ****, $P < 0.0001$). Each image represents the CV-stained biofilms.

protein domain, NosP, involved in the regulation of biofilm formation and dispersal via a multicomponent phosphorelay system (44).

While the exogenous addition of a single dose of NO can disperse a significant portion of biofilms, it was recently found that multiple-dose NO treatment does not improve biofilm dispersal (45). Thus, *P. aeruginosa* biofilms that had been grown for 24 h or 48 h did not show any enhanced dispersal after multiple dosing with the NO donor compound MAHMA NONOate (45). In this study, we hypothesized that after a first exposure to NO, a subpopulation of biofilm cells has an altered signaling pathway and becomes insensitive to NO. We investigated the response of *P. aeruginosa* biofilm cells to successive NO treatments and show that preexposure of biofilms to very low concentrations of NO inhibits the subsequent dispersal response when biofilms are exposed to higher concentrations of NO. Further, the flavohemoglobin encoded by *fhp* and regulated by *fhpR* was found to contribute to the NO pretreatment-induced impairment of dispersal. Finally, we show that this impaired dispersal can be restored by combined treatments with imidazole, a well-known antifungal drug that blocks the NO dioxygenase (NOD) activity of flavohemoglobin. Our findings offer a new perspective on reducing NO compound dosage and improving the use of NO as a biofilm control strategy.

RESULTS

NO-pretreated biofilms show impaired dispersal. Previously, it was found that multiple doses of NO did not significantly increase biofilm dispersal (45). To better understand this phenomenon, the response of *P. aeruginosa* biofilm cells to NO was investigated by using a batch culture multiwell plate biofilm dispersal assay (46). Biofilms with no pretreatment, grown for a total of 6 h 15 min, including a dispersal treatment (15 min), were reduced by 88% after exposure to a dispersing dose, 100 μM, of the NO donor (*Z*)-1-[*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate (spermine NONOate [SP-NO]) compared to control, nondispersed biofilms as seen with crystal violet (CV) staining (Fig. 1A; see Fig. S1A a and c in the supplemental material). According to the confocal image analysis of LIVE/DEAD stained biofilms, 87% of 6-h biofilm live cells were dispersed by the same concentration of NO for 15 min, and there was no significant increase in dead biofilm cells (see Fig. S2 in the

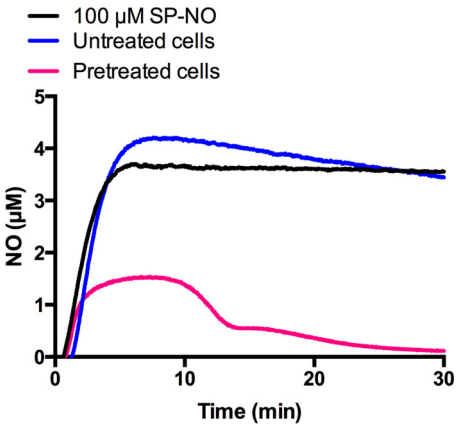

FIG 2 NO-pretreated *P. aeruginosa* cells scavenge free NO. NO levels in solution liberated from 100 μM SP-NO were measured amperometrically in the absence or presence of *P. aeruginosa* cells grown in multiwell plates for 3 h and treated or not with 10 μM SP-NO for a further 3 h.

supplemental material), showing that 100 μM SP-NO did not affect the viability of the biofilm cells. This is comparable to the results of CV staining, where 6-h biofilms were reduced by 88% (Fig. 1A). In this system, biofilms that were pretreated with 10 μM SP-NO (a nondispersing dose) for 3 h before inducing dispersal with 100 μM SP-NO for 15 min had similar biomass with and without the second NO treatment (Fig. 1A; see Fig. S1A b and d). The biomass of these biofilms was also similar to that of control biofilms that were not treated at all during the entire experiment. These data suggest that the bacterial response to a dispersing concentration of NO (100 μM SP-NO) was altered after preexposure to nondispersing concentrations of NO (10 μM SP-NO). However, pretreated biofilms were dispersed when the concentration of the second dose of NO was increased to 200 or 500 μM (Fig. 1B). This suggests that the factor responsible for the pretreatment effect could be titrated away by increasing the effective concentration of NO. Two strains that overproduce alginate were also tested using the pretreatment assay: PDO300, which is a *mucA*22 derivative of PAO1 constructed by allelic exchange (47), and an isogenic Δ*mucA* mutant strain (48). Both strains could be dispersed by a single dose of 100 μM NO, as was observed for the nonmucoid *P. aeruginosa* PAO1 strain. Biofilms of PDO300 and the Δ*mucA* mutant that were preexposed to 10 μM SP-NO were also impaired in their dispersal response after a subsequent exposure to a second, effective dose of NO (100 μM SP-NO) (see Fig. S3 in the supplemental material), which is similar to the wild-type (WT) strain PAO1. In the same system, exposure of biofilms formed by the wild-type PAO1 strain to 10 μM SP-NO did not prevent carbon or oxygen starvation-induced dispersal (see Fig. S4 and S5 in the supplemental material), suggesting that the pretreatment induces a NO-specific response.

NO-scavenging activity is cell associated and not secreted. Because the change in dispersal behavior after pretreatment appeared to be specific to NO-mediated dispersal and could be overcome by using higher levels of the signal molecule, we hypothesized that biofilm bacteria may induce a response to scavenge and reduce NO levels. Instantaneous levels of NO in solution liberated from donor compounds were measured amperometrically using NO-specific electrodes in the presence of various bacterial cultures. The NO donor SP-NO was again used, and at a concentration of 100 μM donor compound, the amount of NO in solution was found to reach a steady state of 3.7 μM after 7 min (Fig. 2). While the addition of untreated *P. aeruginosa* cells had no effect on the release of NO, when NO-pretreated cells were added to the 100 μM SP-NO solution, the amount of NO measured was dramatically reduced (Fig. 2). In contrast, the cell-free supernatant of pretreated cells did not have any impact on NO levels (see Fig. S6 in the supplemental material), suggesting that the NO-scavenging capacity was not due to secreted factors but was associated with intracellular compo-

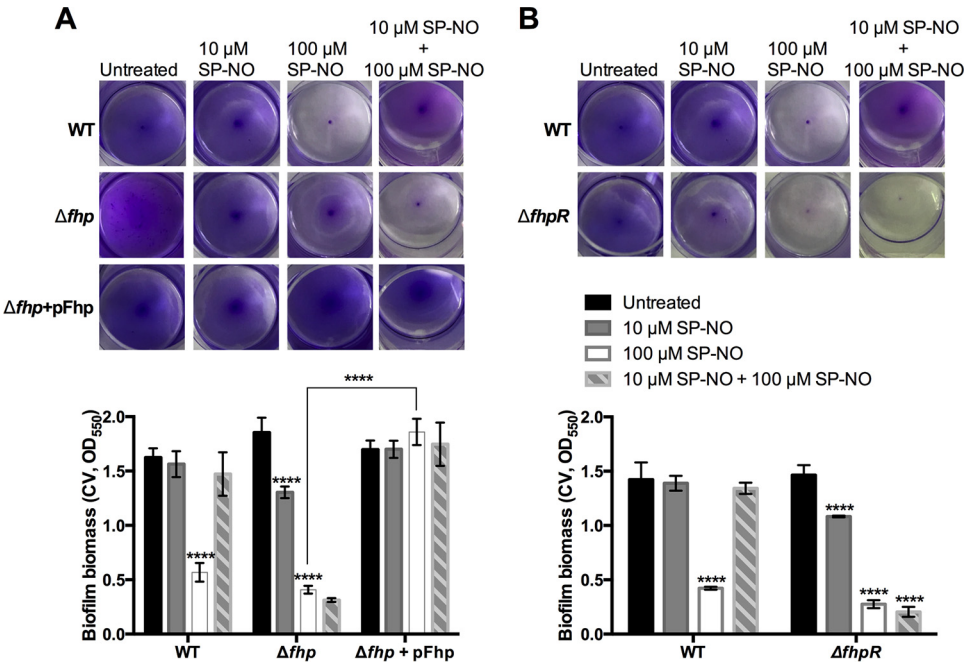

FIG 3 *P. aeruginosa* Δ*fhp* and Δ*fhpR* mutant strains are not affected by NO pretreatment. Biofilms of *P. aeruginosa* WT, Δ*fhp* (A) and Δ*fhpR* (B) transposon mutants, and the Δ*fhp* strain complemented with pFhp plasmid (A) were grown in multiwell plate batch cultures for a total of 6 h 15 min, including or not a pretreatment with 10 μM SP-NO at $t = 3$ h and a dispersal treatment with 100 μM SP-NO at $t = 6$ h. At the end of the incubation, the biofilm biomass was analyzed by CV staining. The error bars indicate standard deviations ($n = 3$). The asterisks indicate statistically significant differences compared to untreated control samples or between samples, as indicated (****, $P < 0.0001$). Each image represents the CV-stained biofilms.

nents. This was also supported by the fact that replacing the supernatants of biofilm cultures did not alleviate the dispersal prevention effect of pretreatment with NO (see Fig. S7 in the supplemental material). Taken together, these results indicate that NO pretreatment induced the production of NO-scavenging factors, which were cell associated and not secreted.

***fhp* and *fhpR* are required for inhibiting dispersal.** In *P. aeruginosa*, several proteins have been characterized that have the ability to prevent nitrosative stress by scavenging NO through reduction reactions, such as the NO reductase NOR (encoded by *norCB*), or oxidation reactions, such as the dioxygenases Fhp, HmgA, and Hpd (49). To determine if these proteins might be involved in scavenging NO and preventing dispersal, transposon mutant strains, including *P. aeruginosa* Δ*fhp*, Δ*norB*, Δ*norC*, Δ*hmgA*, and Δ*hpd*, were tested using the pretreatment assay. In contrast to all of the other mutants tested, the *P. aeruginosa* Δ*fhp* mutant was readily dispersed in response to 100 μM SP-NO despite pretreatment with 10 μM SP-NO (Fig. 3A; see Fig. S8 in the supplemental material). Complementation of the Δ*fhp* mutant restored the wild-type phenotype with regard to the dispersal defect. Further, even in the absence of pretreatment, the complemented strain showed enhanced resistance to 100 μM SP-NO-induced dispersal compared to the wild type (Fig. 3A). This was probably due to the high copy number of the plasmid pUCP22, leading to high expression of Fhp and, hence, NO scavenging. Taken together, these data suggest that Fhp production is the main contributor to the NO pretreatment-induced impairment of dispersal.

The transcriptional activity of the *fhp* promoter is known to be dependent on the regulators FhpR, AsrA, and PA3697 (37, 50). To determine which regulator of *fhp* may be involved in the dispersal impairment, the Δ*fhpR*, Δ*asrA*, and PA3697 transposon mutant strains were tested. The results showed that NO-pretreated biofilms formed by *P. aeruginosa* Δ*fhpR* also lost their defect in dispersal (Fig. 3B). In contrast, the Δ*asrA* and PA3697 transposon mutants were not affected (see Fig. S9 in the supplemental

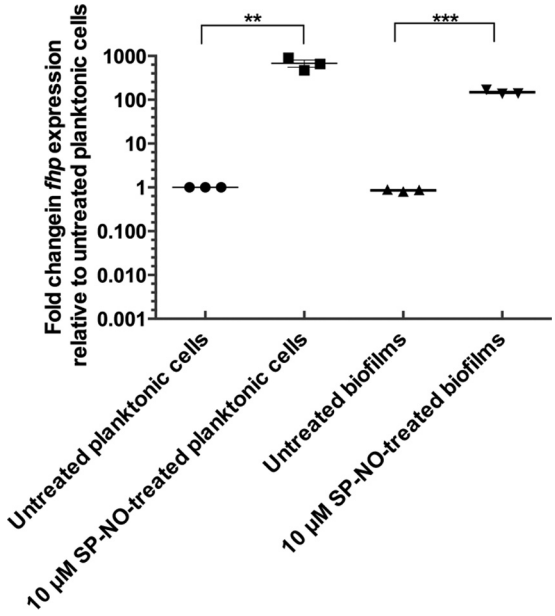

FIG 4 NO pretreatment induces the expression of *fhp*. *P. aeruginosa* PAO1 was grown in multiwell plate batch cultures for 3 h before being treated or not with 10 μM SP-NO. After 15 min, mRNAs were extracted from biofilm and planktonic bacteria, and *fhp* mRNA levels were quantified by qRT-PCR analysis. The data show fold changes in *fhp* transcript levels relative to untreated planktonic cells. The error bars indicate standard deviations ($n = 3$). The asterisks indicate statistically significant differences between samples as indicated (**, $P < 0.01$; ***, $P < 0.001$).

material). This suggests that *fhpR* may play a role in regulating the production of NO-scavenging proteins after exposure to low levels of NO.

Expression of *fhp* increases significantly after exogenous NO pretreatment and moderately over the developmental time course of untreated biofilms. Since previous results indicated that Fhp contributes to the NO pretreatment-induced impairment of dispersal, we hypothesized that *fhp* might be upregulated after exogenous pretreatment stimulation. Quantitative real-time reverse transcription-PCR (qRT-PCR) was used to measure transcription levels of *fhp* in biofilms and planktonic cells with and without NO pretreatment. After 3 h, biofilms and planktonic cells showed similar *fhp* expression levels before exposure to NO (Fig. 4). Expression levels of *fhp* were also similar in 6-h planktonic cells and 6-h biofilms (data not shown). Treatment with 10 μM SP-NO induced a strong increase in *fhp* expression in both 3-h biofilms and planktonic cells, by over 100- and 600-fold, respectively, compared to the untreated controls (Fig. 4), suggesting that *fhp* expression does not happen only in planktonic cells but can also occur in biofilms, which could compromise biofilm control strategies that rely on using NO as a dispersing agent.

To further investigate the role of *fhp* in biofilm development, we then sought to determine its expression dynamics during biofilm formation. Expression levels of *fhp* in biofilms were found to increase by 23.3-fold without any exogenous stimulation in 6-h biofilms compared to 3-h biofilms, suggesting that *fhp* is induced in older biofilms (Fig. 5A). Under these experimental conditions, which were optimized for the fast formation of attached biofilm biomass, dispersal of biofilm cells is known to occur naturally after 8 to 10 h due to the depletion of the carbon source and the onset of starvation (51). Therefore, in order to study the expression of *fhp* in biofilms older than 10 h, biofilms were grown at room temperature with shaking at 120 rpm instead of 37°C at 180 rpm, which allowed the biofilm biomass to increase continuously for over 20 h. By using this system, the average of normalized transcript levels of *fhp* was found to be 6.8-fold higher in 20-h biofilms than in 6-h biofilms (Fig. 5B), although these changes were not statistically significant due to the large standard error. In this system, *fhp* was also induced in 12-, 16-, and 20-h unstimulated biofilms compared to 8-h biofilms (Fig. 5C).

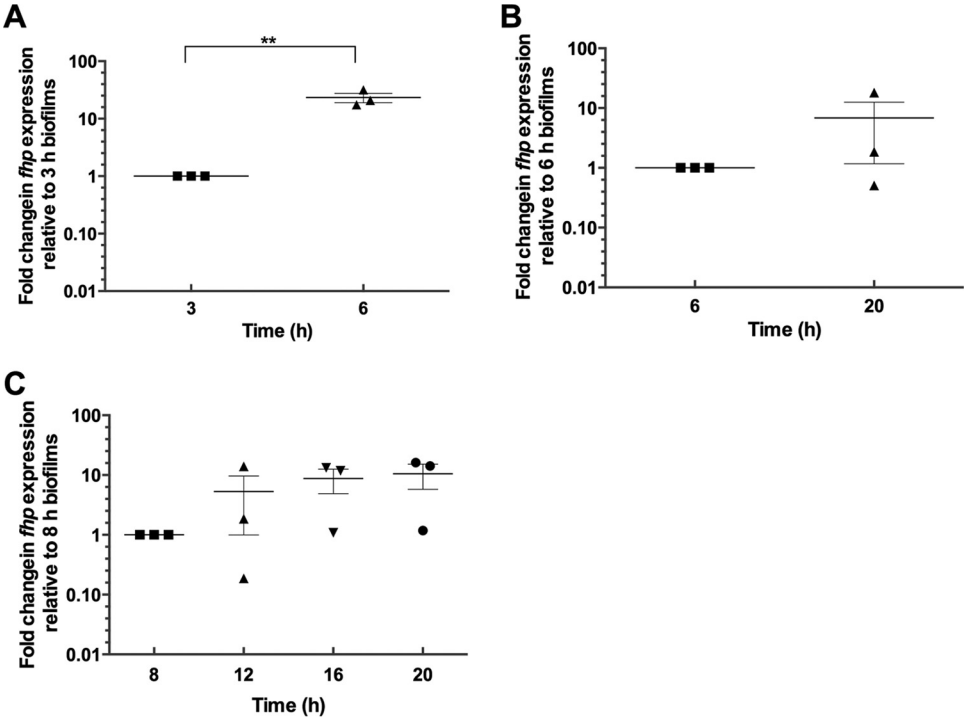

FIG 5 *fhp* expression increases over time during biofilm development. *fhp* mRNA levels were quantified by qRT-PCR analysis and compared at different time points in biofilms grown at 37°C with fast agitation (A) or compared between 6-h biofilms grown at 37°C with fast agitation and 20-h biofilms grown at room temperature with slower agitation, a system that allows continuous increase in biofilm biomass over 20 h (B). (C) Time course analysis of *fhp* mRNA levels in the 20-h biofilm system. The error bars indicate standard errors ($n = 3$). The asterisks indicate statistically significant differences between samples (**, $P < 0.01$).

Expression levels of *fhp* were similar in 20-h biofilms and overnight culture (16-h planktonic cells) (data not shown). Taken together, these results suggest that *fhp* is endogenously induced, albeit to varying degrees, over time during biofilm development depending on the experimental conditions.

In older biofilms, inactivation of *fhp*, but not *fhpR*, leads to enhanced sensitivity to NO-mediated induction of dispersal. Because *fhp* expression appeared to be induced with time in biofilms, we then tested if the dispersal response to NO might be altered in older biofilms. The effects of 10 and 100 μM SP-NO were tested on 20-h biofilms of *P. aeruginosa* wild-type and isogenic Δ*fhp* and Δ*fhpR* mutant strains. In this biofilm assay, a low dose of NO generated from 10 μM SP-NO dispersed 86% of the biofilm biomass in the *P. aeruginosa* Δ*fhp* mutant, while only 10% and 26% of biofilms formed by *P. aeruginosa* wild type and Δ*fhpR* were dispersed by 10 μM SP-NO (Fig. 6A). In the presence of 100 μM SP-NO, 90% of both wild-type and Δ*fhp* biofilms were removed, and Δ*fhpR* biofilms were dispersed by about 61%. This suggests that 20-h biofilms of Δ*fhp* were more sensitive to NO signals than those of the wild-type and Δ*fhpR* mutant strains. qRT-PCR analysis of 20-h wild-type and Δ*fhpR* biofilms did not show any significant differences in *fhp* mRNA levels between the strains (Fig. 6C), suggesting that *fhpR* was not necessary for the nonstimulated, endogenous induction of *fhp* in 20-h biofilms. However, in contrast to the wild-type strain, the Δ*fhp* and Δ*fhpR* mutant strains still retained their dispersal capacity after pretreatment stimulation with an initial low dose of NO in the 20-h biofilm system (Fig. 6B), which correlates with the results previously obtained with the 6-h biofilm system (Fig. 1A).

Imidazole blocks the NO pretreatment effect and enhances dispersal by inhibiting flavohemoglobin. Because the production of Fhp impaired NO-mediated dispersal, we hypothesized that inhibiting the NOD activity of Fhp could restore dispersal in pretreated biofilms. One such candidate inhibitor compound is imidazole, which has

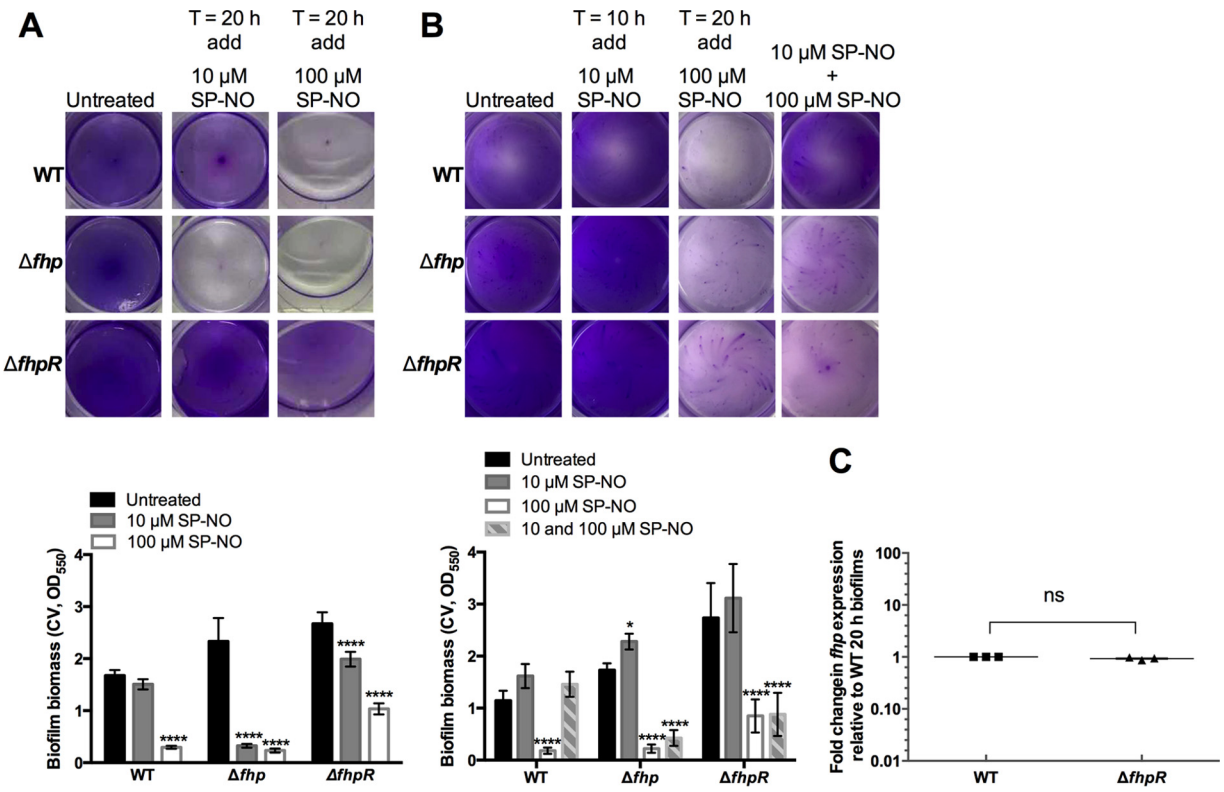

FIG 6 (A) The Δ*fhp* mutant strain is more sensitive to NO-mediated induction of dispersal than the wild-type strain and the Δ*fhpR* mutant in 20-h biofilms. Biofilms of *P. aeruginosa* WT and the Δ*fhp* and Δ*fhpR* transposon mutant strains grown in multiwell plate batch cultures at room temperature for 20 h were treated with 10 μM or 100 μM SP-NO for 15 min before CV staining. Experimental details are shown in Fig. S1B in the supplemental material. The error bars indicate standard deviations ($n = 3$). The asterisks indicate statistically significant differences compared to untreated control samples (****, $P < 0.0001$). Each image represents the stained biofilms. (B) Δ*fhp* and Δ*fhpR* mutant strains still retain their dispersal capacity after pretreatment with an initial low dose of NO in the 20-h biofilm system. Biofilms of *P. aeruginosa* WT and Δ*fhp* and Δ*fhpR* transposon mutant strains were grown in multiwell plate batch cultures for a total of 20 h 15 min, including or not a pretreatment with 10 μM SP-NO at $t = 10$ h and a dispersal treatment with 100 μM SP-NO at $t = 20$ h. At the end of the incubation, the biofilm biomass was analyzed by CV staining. Experimental details are shown in Fig. S1C in the supplemental material. The error bars indicate standard deviations ($n = 3$). The asterisks indicate statistically significant differences compared to untreated control samples (*, $P < 0.1$; ****, $P < 0.0001$). Each image represents the stained biofilms. (C) No significant differences were found in *fhp* mRNA levels between 20-h biofilms formed by *P. aeruginosa* wild type and the Δ*fhpR* mutant. The error bars indicate standard errors ($n = 3$). ns, no significant difference.

been previously reported to hinder the NOD activity of bacterial and fungal flavohemoglobins by coordinating and constraining their heme (52). In the current study, imidazole was tested for its ability to inhibit purified flavohemoglobin and flavohemoglobin produced in *P. aeruginosa* biofilms. The NO consumption activity of purified histidine-tagged Fhp from *P. aeruginosa* with and without imidazole treatment was investigated by using NO-specific electrodes and an appropriate buffer system to study NOD kinetics (52). As expected, the addition of 5 nM Fhp purified from *P. aeruginosa* dramatically reduced the amount of NO released from 100 μM SP-NO (Fig. 7A). In contrast, Fhp that had been treated with imidazole did not have any effect on the release of NO. Furthermore, truncated Fhp proteins that lacked the flavin-binding or heme-binding domain both lost the ability to scavenge NO (see Fig. S10 in the supplemental material).

In the absence of imidazole, 6-h biofilms pretreated with a low dose of NO (10 μM SP-NO) were impaired in their dispersal response to 100 μM SP-NO, as expected. However, the addition of 5 mM imidazole for 15 min prior to the final treatment with 100 μM SP-NO restored the dispersal response, resulting in the detachment of 64% of biofilms (Fig. 7B). Treatment with imidazole alone showed only a 12% reduction in biofilm biomass. Overall, these data strongly suggest that imidazole can inhibit the flavohemoglobin function in NO-pretreated biofilms and enhance biofilm dispersal.

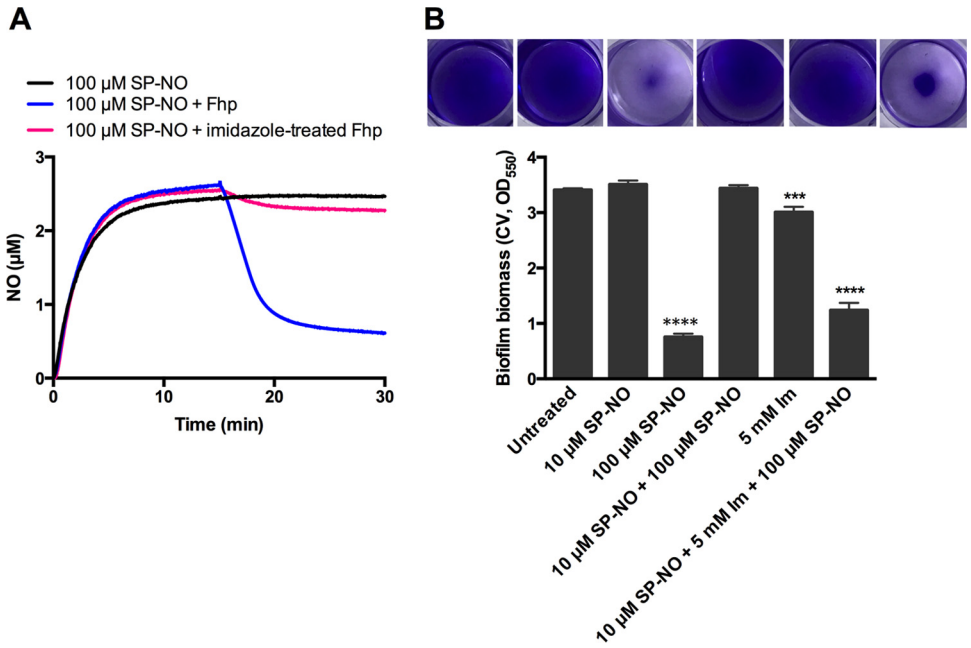

FIG 7 Imidazole can inhibit the NOD activity of Fhp and restore the dispersal response of *P. aeruginosa* biofilms pretreated with NO. (A) NO released from 100 μM SP-NO in the absence and presence of Fhp and imidazole was measured amperometrically in 100 mM sodium phosphate buffer, pH 7.0, containing 0.3 mM EDTA, 100 μM NADPH, and 1 μM FAD at room temperature. Fhp (5 nM), either untreated or treated with imidazole (5 mM), was added 15 min after adding SP-NO. (B) *P. aeruginosa* biofilms were grown in multiwell plate batch cultures for 6 h. Then, the biofilms were treated or not with 5 mM imidazole (Im) for 15 min, after which time dispersal was induced with 100 μM SP-NO for 15 min before quantifying the attached biomass by CV staining. For the pretreatment experiments, biofilms were grown for 3 h, exposed to 10 μM SP-NO, and incubated for a further 3 h (6 h total) before being exposed to imidazole and NO, followed by assessment of the total biofilm biomass by CV staining. The error bars indicate standard deviations ($n = 3$). The asterisks indicate statistically significant differences compared to untreated control samples (***, $P < 0.001$; ****, $P < 0.0001$). The images show the CV-stained biofilms.

We further investigated whether the addition of imidazole could improve the dispersal response to NO in *P. aeruginosa* biofilms by inhibiting flavohemoglobin produced naturally during biofilm development rather than after induction with exogenous NO. In this experiment, biofilms were not preexposed to NO but were treated with imidazole and a low dose of NO (10 μM SP-NO) successively. It was found that combined treatments of imidazole (5 or 10 mM) and 10 μM SP-NO failed to disperse 6-h *P. aeruginosa* biofilms (Fig. 8A). In contrast, in 20-h biofilms, although 10 μM SP-NO alone also had no impact on the biofilms, combined treatments of imidazole (5 and 10 mM) and 10 μM SP-NO dispersed about 88% of the biofilms (Fig. 8B).

DISCUSSION

An endogenous mechanism to limit biofilm dispersal. In this work, an experimental system was designed to test the effect of successive exposures to NO signals on biofilm dispersal. We were able to replicate the previously observed attenuation of dispersal responses after multiple treatments with NO by first exposing biofilms to a low, nondispersing concentration, followed by NO concentrations that would normally disperse biofilms (Fig. 1). By using this system, we found that the pretreatment effect was due to strong induction of *fhp*, encoding the NO-scavenging flavohemoglobin, probably after direct sensing of NO by FhpR. Thus, this work has uncovered a novel role for the flavohemoglobin Fhp and the regulator FhpR, which, in addition to protecting bacteria from nitrosative stress, are also involved in mediating dispersal responses to NO signals. Interestingly, the pretreatment-induced repression of dispersal appeared to be specific for the NO-mediated signaling pathway, while dispersal responses induced by oxygen or nutrient limitation were not affected by NO pretreatment (see Fig. S4 and S5 in the supplemental material). This clarifies the fact that, despite the observation that

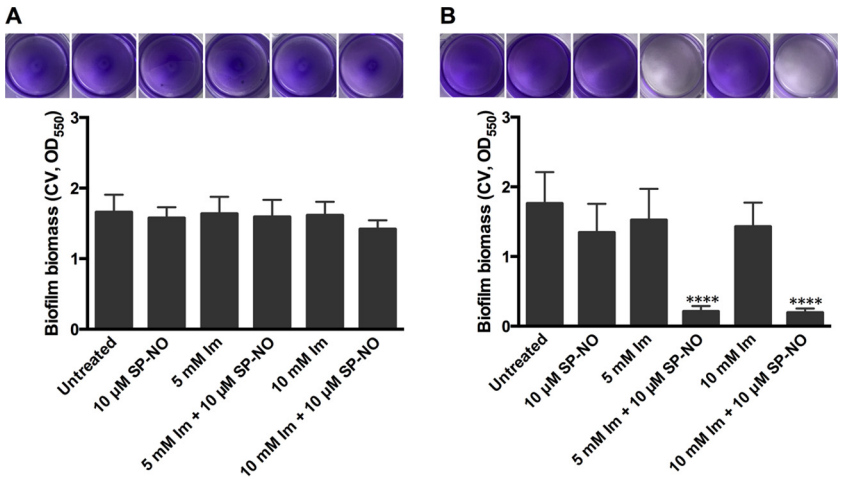

FIG 8 Imidazole enhances the sensitivity of the dispersal response to NO in 20-h biofilms. Biofilms were grown in multiwell plate batch cultures for 5 h (A) or 19 h (B) and subsequently treated with 5 and 10 mM imidazole for 1 h prior to addition of 10 μM SP-NO for 15 min. The error bars indicate standard deviations ($n = 3$). At the end of the incubation, the biofilm biomass was analyzed by CV staining. The asterisks indicate statistically significant differences compared to untreated control samples (****, $P < 0.0001$). The images show the CV-stained biofilms.

all three dispersal responses to oxygen depletion, starvation, and NO operate through modification of intracellular c-di-GMP concentrations and require the periplasmic protease LapG (53), the oxygen- and nutrient-controlled dispersal pathways are independent of NO signaling and, for instance, do not involve production of Fhp.

The results presented here show that the NO dispersal pathway can be modulated by Fhp activity, the production of which is induced in response to NO exposure, through the regulator FhpR. Fhp has a dioxygenase function that converts NO to nitrate (NO_3^-) (37, 54), and this reaction is dependent on NADPH, flavin adenine dinucleotide (FAD), and oxygen (55). Our results showed that the expression level of *fhp* was highly induced by a nondispersing dose of NO (10 μM SP-NO) in both biofilms and planktonic cells. The induction of *fhp* after exposure to low levels of NO was quite surprising, given that a 1.8-fold downregulation of *fhp* in response to NO has been previously observed in *P. aeruginosa* biofilms (11). However, the experimental conditions in the previous studies were different, as biofilms were grown for 5 days and exposed to the NO donor sodium nitroprusside for 1 h compared to biofilms treated with NO after 3 h, that is, almost immediately after cell attachment in the current work. Thus, it is possible that in more mature biofilms, *fhp* exhibits different expression and regulation profiles. This is supported by our observation that *fhp* expression was higher in 20-h biofilms than in 6-h biofilms.

In *P. aeruginosa*, NO could also be reduced to N_2O by NOR (56, 57), although this pathway was not necessary for the NO scavenging observed in the 6-h biofilms. The expression of the denitrification genes *norB* and *norC* occurs under anaerobic or low-oxygen conditions (37). This suggests that the biofilms studied here were not experiencing oxygen limitation based on the incubation conditions.

We further demonstrated that the regulator *fhpR* was required for the pretreatment-induced impaired dispersal. *fhp* and *fhpR* are adjacent and share a bidirectional promoter region, which is independent of DNR or ANR (37). FhpR is a homologue of NorR in *E. coli* that regulates the production of flavorubredoxin (39, 40). It was previously reported that the *fhp* promoter is dependent on two additional regulators, AsrA and PA3697 (50), although neither appeared to be required in our assays for pretreatment-induced impaired dispersal (see Fig. S9 in the supplemental material). Interestingly, *fhpR* was not required for these effects in the 20-h biofilms, which suggests that an additional regulatory mechanism controls *fhp* expression in the older, 20-h biofilms.

Flavohemoglobin is distributed broadly among prokaryotic and eukaryotic microorganisms (58). In *E. coli* and *Staphylococcus aureus*, the expression of genes encoding flavohemoglobins has been shown to be stimulated by NO and nitrosative stresses (59, 60). Therefore, it may protect mixed-community biofilms found in natural and engineered systems from NO-induced dispersal.

Flavohemoglobin may also play a role across a biofilm-host interface. NO, which at high concentrations can cause nitrosative damage to bacteria, is known to be produced by NO synthases in infected host tissues in both mammals and plants (61, 62). In the context of a symbiotic relationship between bacteria and a host, flavohemoglobin could potentially confer resistance to the host immune defenses. Thus, in the squid-*Vibrio* symbiosis, Hmp has been shown to alleviate nitrosative stress in the *Vibrio fischeri* symbiont and to allow its successful colonization of the host while other invading bacterial species are excluded (63, 64).

Overall, our data have uncovered an intrinsic mechanism by which bacteria can modulate their response to NO-induced dispersal. In the context of biofilm development, NO production from the deep anaerobic zones of mature microcolonies has been linked to the death of a subpopulation of cells and the formation of hollow voids within microcolonies (19). Induction of *fhp* expression could be a potential strategy by which biofilms may limit the extent of biofilm dispersal induced by endogenously produced NO in mature biofilms, e.g., in microcolonies undergoing seeding dispersal. Future studies in our laboratory will investigate the dynamics and spatial localization of *fhp* expression during biofilm development and dispersal.

Combined treatments with NO and Fhp inhibitors to improve biofilm control. Previous studies have shown that imidazole compounds can inhibit NOD function by coordinating heme iron and "fitting" within the heme pocket, since they have bulky aromatic substituents (65–68). Here, imidazole was found to block the NO pretreatment-induced impairment of dispersal and to work synergistically with NO to disperse biofilms at very low concentrations of NO donors. Thus, it is possible that imidazole or related compounds could improve the efficacy of NO as a dispersing agent for biofilms formed by a broad range of microorganisms. It was particularly surprising that in the presence of imidazole, 20-h biofilms were able to disperse in response to 10 μM SP-NO while 6-h biofilms did not disperse in response to such a low dose of NO (Fig. 8). There are several possible explanations for this observation. First, cells in younger biofilms (6-h biofilms) have additional mechanisms of NO defense, in addition to Fhp, that are capable of scavenging NO to some level and that could be absent in 20-h biofilms, while older biofilms (20-h biofilms) may rely mostly on Fhp. Alternatively, older biofilms may have an altered pathway for inducing dispersal in response to lower levels of NO rather than a pathway for scavenging NO. Further experiments will need to focus on these possibilities to better define the mechanisms of NO response for younger and older biofilms.

Naturally occurring and synthetic derivatives of imidazole have been well documented (69–71). For example, sponges of the genera *Leucetta* and *Clathrina* are rich sources of imidazole alkaloids (72–74). In the future, it will be interesting to investigate the combined effects of low doses of NO and different imidazole derivatives with low toxicity and high bioavailability on biofilms formed by single and multiple species.

In conclusion, this study has identified a mechanism to explain the previous observations that multiple doses of NO do not improve biofilm dispersal. A novel role for the NO-scavenging flavohemoglobin Fhp has been identified, where Fhp is induced in an NO-dependent fashion by FhpR in younger biofilms and subsequently impairs the dispersal responses to NO. Further, this work highlights an innovative strategy for potentiating or synergizing the activity of NO as a biofilm control agent by inhibiting natural scavenging mechanisms. Our study offers a new perspective on improving the use of NO for biofilm control in both clinical and industrial settings.

MATERIALS AND METHODS

Bacteria and growth conditions. The *P. aeruginosa* WT strain PAO1 (75); strain PDO300, which is a *mucA*22 derivative of PAO1 constructed by allelic exchange (47); and an isogenic Δ*mucA* mutant strain (48) were used in this study. *P. aeruginosa* mutant strains containing a transposon Tn*5*-derived insertion element (Tc^r) in key genes involved in NO detoxification, which were obtained from the University of Washington *P. aeruginosa* PAO1 transposon mutant library (supported by grant number NIH P30 DK089507), were also used: Δ*fhp* (PA2664), strain PW5458 *fhp*-F06::IS*lacZ*/hah (an influenza hemagglutinin [HA] epitope and a hexahistidine motif); Δ*fhpR* (PA2665), strain PW5460 *fhpR*-H11::IS*phoA*/hah; Δ*norB* (PA0524), strains PW1961 *norB*-A04::IS*phoA*/hah and PW1962 *norB*-A11::IS*phoA*/hah; Δ*norC* (PA0523), strains PW1959 *norC*-B02::IS*lacZ*/hah and PW1960 *norC*-H04::IS*phoA*/hah; Δ*hmgA* (PA2009), strain PW4489 *hmgA*-C03::IS*phoA*/hah; Δ*hpd* (PA0865), strains PW2577 *hpd*-H01::IS*lacZ*/hah and PW2578 *hpd*-H02::IS*lacZ*/hah; Δ*asrA* (PA0779), strains PW2411 *asrA*-A07::IS*lacZ*/hah, PW2413 *asrA*-G11::IS*lacZ*/hah, and PW2414 *asrA*-C03::IS*lacZ*/hah; and ΔPA3697, strains PW7256 PA3697-H04::IS*lacZ*/hah, PW7257 PA3697-C03::IS*lacZ*/hah, and PW7258 PA3697-D09::IS*phoA*/hah (76). For experiments involving the transposon mutants, the isogenic wild-type strain MPAO1 was used (77); no difference in dispersal behavior or response to NO between strains PAO1 and MPAO1 has been observed. Bacteria were routinely grown in Luria-Bertani (LB)-Miller broth (BD Difco) overnight at 37°C, with agitation at 200 rpm for 16 h to prepare the cells for experiments.

NO donor compounds. The NO donor compound SP-NO was obtained from Cayman Chemical. SP-NO spontaneously dissociates to liberate 2 moles of NO per mole of the parent compound, with half-lives of 39 min and 230 min at 37°C and 22 to 25°C, respectively (pH 7.4) (78, 79). Aliquots of 100 mM stock solutions of SP-NO dissolved in 10 mM NaOH (Merck) were kept at −20°C for up to 3 months and thawed only once for each experiment. Further dilutions were freshly made in 10 mM NaOH and used immediately.

Biofilm dispersal assays. Biofilms were grown as previously described (46) with some modifications. Briefly, overnight cultures of *P. aeruginosa* PAO1 wild type and mutants were diluted 200-fold to an optical density at 600 nm (OD_{600}) of 0.003 in M9 minimal medium (containing 9 mM NaCl, 22 mM KH_2PO_4, 48 mM Na_2HPO_4, 19 mM NH_4Cl, 2 mM $MgSO_4$, 100 μM $CaCl_2$, and 0.4% glucose, pH 7.0), which was freshly prepared for every experiment. One milliliter of the diluted culture was added to 24-well microtiter plates (Nunc, Thermo Fisher Scientific), and the plates were incubated on an orbital shaker at 180 rpm for 6-h biofilms, which were cultivated at 37°C, or at 120 rpm for 20-h biofilms, which were cultivated at room temperature. After 6 h or 20 h growth, SP-NO was added to the cultures at a final concentration of 100 μM to induce biofilm dispersal, and the plates were incubated for a further 15 min. For the untreated control, an equal amount of 10 mM NaOH was added. After the final incubation, the biofilm biomass was analyzed by CV staining. Briefly, biofilms grown on the interior surfaces of the multiwell plate wells were washed with phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4, and 10 mM Na_2HPO_4 (pH 7.4) to remove loosely attached bacteria before staining for 20 min with 1 ml of 0.1% CV solution made by diluting a 1% CV aqueous solution (Sigma) 1:10 in Milli-Q water. The stained biofilms were washed twice with 1 ml PBS, and the remaining CV was dissolved in 1 ml of absolute ethanol. The biofilm biomass was quantified by measurement of the absorbance at 550 nm (OD_{550}) in an Infinite Pro2000 microplate reader (Tecan). All biofilm assays were performed in independent triplicate experiments.

For microscopy analysis, *P. aeruginosa* PAO1 wild-type biofilms grown in multiwell plate batch cultures for 6 h and subsequently left untreated or treated with 100 μM SP-NO for 15 min were rinsed once with PBS before being stained with the LIVE/DEAD BacLight bacterial viability kit reagents (Molecular Probes, Inc.) according to the manufacturer's procedure. One microliter of each of the two components was mixed thoroughly in 1 ml of PBS, and then 0.5 ml of this solution was added into each well and allowed to incubate at room temperature in the dark for 15 min. Images of untreated and NO-treated biofilms were acquired using confocal laser scanning microscopy (CLSM) (Carl Zeiss Microscopy; LSM 780). Biofilm quantification was performed using the IMARIS software package (Bitplane AG).

To test successive exposures to NO treatments, *P. aeruginosa* biofilms that had been grown in multiwell plates as described above for 3 h received a pretreatment with 10 μM SP-NO before incubating the plates for another 3 h. After a total of 6 h, the biofilms were treated or not with 100 μM SP-NO for 15 min to induce dispersal before quantifying the biofilm biomass by CV staining.

For experiments involving the alginate-overproducing strains, biofilms of all three strains, including PAO1 wild type, PDO300, and Δ*mucA* mutant were grown in M9 medium supplemented with 2% Casamino acids, where the higher amount of Casamino acids was necessary for the mucoid strains to form biofilms in our experimental conditions. Exposure to NO donors and biofilm biomass analysis by CV staining were carried out as described above.

The biofilm assay was also used to test the effect on biofilm dispersal of the flavohemoglobin inhibitor imidazole (Sigma-Aldrich), which was prepared in Milli-Q water at different concentrations. Biofilms that had been pretreated with 10 μM SP-NO after 3 h growth (6-h biofilms) were then treated with 5 mM imidazole for 15 min prior to addition of 100 μM SP-NO to induce dispersal. Biofilms that had been grown for 5 h or 19 h without pretreatment were treated with 5 or 10 mM imidazole for 1 h prior to addition of 10 μM SP-NO. The biofilm biomass was quantified by CV staining as described above.

Amperometric measurements of NO. The concentration of NO liberated from SP-NO was measured amperometrically by using a TBR1025 free radical analyzer (World Precision Instruments) equipped with a NO-specific ISO-NOP 2-mm electrode with a detection range from 1 nM to 100 μM and calibrated by using MAHMA NONOate (Cayman Chemical) as the NO donor.

NOD activity of purified Fhp was also assessed amperometrically by following a protocol previously described by Gardner and Gardner (80). NO was released from 100 μM SP-NO in a NOD activity buffer solution consisting of 100 mM Na_3PO_4, pH 7.0, 0.3 mM EDTA, 100 μM NADPH (Cayman Chemical), and 1 μM FAD (Sigma-Aldrich) at room temperature; according to the method, NADPH and FAD were added before adding SP-NO. Fhp, imidazole-treated Fhp, and truncated Fhp were added 15 min after adding SP-NO. Imidazole-treated Fhp was obtained by incubating 500 nM Fhp with 500 mM imidazole for 1 h at room temperature. The mixture was added to NOD activity buffer in the presence of FAD, NADPH, and NO. The final concentrations of Fhp and imidazole were 5 nM and 5 mM, respectively.

Generation of the complemented strain. An *fhp* complementation plasmid was constructed by amplifying the *fhp* gene, including 156 bp of the promoter-containing upstream region, with the primers *fhp*_for/*fhp*_rev (see Table S1 in the supplemental material) using *Pfu* polymerase (Thermo Fisher Scientific) and ligating it into EcoRI and BamHI (New England BioLabs) double-cut plasmid pUCP22. The resultant plasmids were transformed into the *E. coli* donor strain DH5α and transformed into the transposon mutant Δ*fhp* (PW5458 *fhp*-F06::IS*lacZ*/hah).

qRT-PCR. cDNA was reverse transcribed from 0.5 μg of total RNA using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's protocols. The cDNA was then used as the template for qRT-PCR in a real-time PCR system using SYBR green with the Rox detection system (Kapa Biosystems) and primers, including *fhp*_RT_for/*fhp*_RT_rev for *fhp* and *rpoD*_RT_for/*rpoD*_RT_rev for the housekeeping gene *rpoD*. The primers are listed in Table S1 in the supplemental material. Each sample was independently tested three times and assayed in triplicate during each run. Relative gene expression with respect to *rpoD* mRNA transcript levels was calculated using the $2^{-\Delta\Delta CT}$ method (81).

Fhp protein purification. Cloning and protein expression were carried out at the Protein Production Platform (PPP) at Nanyang Technological University (NTU). Targets of *fhp* (1,175 bp), truncated *fhp* without a flavin-containing domain (422 bp), and truncated *fhp* without a heme-binding domain (716 bp) were amplified using primers Fhp-f7852/Fhp-r7876, Fhp-f7852/Fhp-r7880, and Fhp-f7854/Fhp-r7877 (see Table S1 in the supplemental material). All cloning was performed in 96-well systems using ligation-independent cloning (LIC) technology in *E. coli* (82, 83). Histidine-tagged-protein production was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) and analyzed on SDS-PAGE gels. Clones with acceptable levels of expression were selected for large-scale expression and purification. During the purification steps, cell lysates were loaded on immobilized-metal affinity chromatography (IMAC) columns (1 ml Ni-nitrilotriacetic acid [NTA] Superflow [Qiagen]) and were washed. His-tagged proteins were eluted with 500 mM imidazole, collected, and stored in sample loops on the system and then injected into gel filtration (GF) columns (Superdex 200 HR 26/60 [GE Healthcare]), which separated imidazole from the proteins. Elution peaks were collected in 2-ml fractions and analyzed on SDS-PAGE gels. Relevant peaks were pooled, and tris(2-carboxyethyl)phosphine (TCEP) was added to a total concentration of 2 mM. Purified proteins were kept in buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 10% (vol/vol) glycerol, and 2 mM TCEP. The final protein batch was then aliquoted into smaller fractions, frozen in liquid nitrogen, and stored at −80°C.

Statistical analysis. Multivariate analyses were performed using *t* tests, one-way analysis of variance (ANOVA), and two-way ANOVA, followed by the Sidak posttest for individual comparisons.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01832-17.

SUPPLEMENTAL FILE 1, PDF file, 3.9 MB.

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We declare no conflict of interest.

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