Nitric oxide and iron signaling cues have opposing effects on biofilm development in *Pseudomonas aeruginosa*

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ABSTRACT

While both iron and nitric oxide (NO) are redox-active environmental signals that have been shown to regulate biofilm development, their interaction and roles in regulating biofilms have not been fully elucidated. In this study, exposure of *Pseudomonas aeruginosa* biofilms to exogenous NO inhibited the expression of iron acquisition related genes and the production of the siderophore pyoverdine. Further, supplementation of the culture medium with high levels of iron (100 µM) counteracted NO induced biofilm dispersal by promoting the rapid attachment of planktonic cells. In the presence of iron, biofilms were found to disperse transiently to NO, while the freshly dispersed cells reattached rapidly within 15 min. This effect was not due to scavenging of NO by free iron, but rather involved a cellular response induced by iron that led to elevated production of the exopolysaccharide Psl. Interestingly, most Psl remained on the substratum after treatment with NO, suggesting that dispersal involved changes in the interactions between Psl and *P. aeruginosa* cells. Taken together, our results suggest that iron and NO regulate biofilm development via different pathways, both of which include regulation of Psl-mediated attachment. Moreover, the addition of an iron chelator worked synergistically with NO in the dispersal of biofilms.

IMPORTANCE: Nitric oxide (NO), which induces biofilm dispersal, is a promising strategy for biofilm control in both clinical and industrial contexts. However, competing environmental signals may reduce the efficacy of NO. The results presented here suggest that the presence of iron represents one such environmental cue that could antagonize the activity of NO as a biofilm dispersing agent. Based on this understanding, we developed a strategy to enhance dispersal by combining NO with an iron scavenging agent. Overall, this study links two important environmental signals, iron and NO, with their roles in biofilm development and suggests new ways for improving the use of NO in biofilm control strategies.

INTRODUCTION

Biological life cycle transitions are often regulated by the interplay between genetic elements and chemical or environmental cues (1). Understanding these interactions may allow for a better control of developmental processes. In bacterial biofilms, the opposing stages of attachment and dispersal are both controlled by a number of external cues and a network of specific genes. Attachment of bacterial cells onto biotic or abiotic surfaces is mediated by extracellular polymeric substances (EPS) that include adhesive proteins and polysaccharides as well as eDNA (2). The opportunistic pathogen and model biofilm forming organism *Pseudomonas aeruginosa*, produces three types of exopolysaccharides: alginate, Pel and Psl, as well as several proteins that have been shown to be involved in biofilm formation. The adhesin CdrA strongly binds Psl and can anchor cells to the EPS matrix or when secreted crosslink fiber-like Psl strands, thus stiffening the gel-like EPS matrix (3). In *P. aeruginosa*, alginate, Pel and Psl are partly regulated by bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), an intracellular secondary messenger conserved across bacterial species (4, 5). In many bacteria, c-di-GMP levels are controlled by multiple enzymes (phosphodiesterases (PDEs) and diguanylate cyclases (DGCs)), some of which are associated with sensory domains (e.g., PAS domain) capable of responding to extracellular stimuli including environmental cues (e.g., oxygen and redox conditions, light and starvation) as well as cell-to-cell signals (4). While high levels of c-di-GMP, via interaction with transcriptional regulators and direct effectors, usually promote attachment, lower intracellular levels downregulate attachment, induce the expression of motility genes and trigger dispersal.

There are a variety of environmental signals that can induce biofilm dispersal. For example, biofilm dispersal can be triggered by low levels of nitric oxide (NO) (6, 7), oxygen depletion (8, 9), changes in temperature (10) as well as changes in iron levels and nutrient availability (11-14).

Among these cues, NO has attracted particular interest as its role in biofilm dispersal that appears to be conserved across bacterial species. Thus, several promising strategies have been developed to deliver NO and disperse antimicrobial-resistant biofilms that could find applications across a range of industrial and clinical settings (15). NO is a hydrophobic molecule and a highly reactive free radical (16). At low, non-toxic concentrations (nanomolar range) NO induces biofilm dispersal, while higher concentrations may cause nitrosative damage to bacterial cells. In *P. aeruginosa*, NO disperses biofilms through stimulation of phosphodiesterase activity, resulting in decreased intracellular c-di-GMP concentrations and involves the periplasmic protease LapG (7, 17). Several sensors of NO have been identified, including a newly characterized heme-binding sensor protein, NosP, that is involved in regulating biofilm dispersal in *P. aeruginosa* and is highly conserved among bacteria (18). While the exogenous addition of NO can disperse a significant portion of biofilms, the addition of NO generally does not disperse all of the biofilm (6). We have recently shown that the non-dispersing cells become insensitive to NO as a consequence of flavohemoprotein production, which scavenges NO (19).

NO can bind to most transition metals (20), of which, iron is one of the best understood. For example, NO can bind to heme sensors, affect cytochromes or iron-sulfur clusters (21). Interestingly, iron has been shown to impact biofilm developmental processes, where low or high iron concentrations can inhibit or increase biofilm formation, respectively. Thus, iron and NO have opposing activities. However, the direct link between iron and NO in the regulation of biofilms remains poorly understood. Iron is an essential nutrient to sustain bacterial growth and bacteria have evolved several strategies for iron acquisition and uptake (22), which may be especially important in conditions of high cellular density such as biofilms. Mature biofilms exhibit gene expression profiles consistent with iron limitation (23). Previous studies have

92 reported that iron availability controls biofilm formation through several mechanisms, including
93 modulating quorum sensing (QS) cell-cell signaling, stimulating DNA release, or enhancing the
94 production of Psl polysaccharides (13, 24, 25). Generally, under iron-limiting conditions *P.*
95 *aeruginosa* does not form biofilms or only forms flat, unstructured biofilms (13, 26). In contrast,
96 under iron-replete conditions, biofilm formation is increased (14, 24). Further, pyoverdine
97 production is reduced in *P. aeruginosa* cells with lower c-di-GMP levels (27-29). Pyoverdine is a
98 high-affinity siderophore produced by *P. aeruginosa* to acquire iron in an iron-limiting
99 environment (30-32). The mechanisms regulating these effects remain to be fully elucidated and,
100 to date, no c-di-GMP-dependent receptor involved in *pvd* transcription has been identified.
101 Moreover, *P. aeruginosa* Δ*pvdA*, Δ*pvdS* and Δ*fpvA* mutant strains, defective in genes important
102 for pyoverdine synthesis, signaling and uptake (33, 34), were shown to form thin layer biofilms
103 and for the Δ*pvdA* mutant, the biofilm mushroom-like structure were restored when pyoverdine
104 was exogenously added (34). Iron may also affect biofilm formation through the QS signaling
105 pathway. The parental strain forms biofilms poorly under iron limiting condition, while the
106 structured, mushroom-like biofilm formation was largely restored in the *rhlI* mutant (35).
107 Moreover, a recent study showed that in *P. aeruginosa*, high iron (50 and 100 μM $FeCl_3$)
108 promoted Psl production and induced biofilm formation (24). Psl was also found to bind both
109 ferrous and ferric iron and store iron to further induce Psl-dependent biofilm formation (24).
110
111 In this study, transcriptomic analysis of the NO mediated dispersal response was performed to
112 better understand the physiological changes induced by NO. NO treated cells had reduced
113 expression of genes for the synthesis of pyoverdine and the lower-affinity siderophore pyochelin
114 (36) as well as other iron acquisition related genes. Thus, a potential link between iron
115 acquisition and NO mediated dispersal was further explored. Supplementation of the culture

medium with high levels of iron overrode NO induced biofilm dispersal by promoting the rapid attachment of planktonic cells, which was linked to the production of Psl. In contrast, the dispersal response appeared to involve changes in Psl mediated attachment of *P. aeruginosa* cells. Finally, addition of the iron chelator 2,2'-Bipyridine (Bipy) showed a synergetic effect with NO in dispersing biofilms. Simultaneous treatment of biofilms with NO and an iron chelator could enhance biofilm dispersal in environments where high iron levels might inhibit the ability of NO to disperse biofilms.

RESULTS

NO inhibits expression of iron acquisition related genes and pyoverdine production

To elucidate the molecular pathway of NO induced dispersal, this study compared transcriptomic profiles of *P. aeruginosa* untreated, planktonic and biofilm cells to those of NO induced dispersed bacteria as well as cells remaining within biofilm structures after treatment with the NO donor, spermine NONOate (SP-NO). Methods of the transcriptomic experiment are described in the supplemental material Text S1. The results showed that the expression levels of most iron acquisition related genes in NO treated biofilms and dispersed cells were lower compared to untreated cells (Supplemental material Table S1 and S2). Several extracytoplasmic function sigma factors (ECF-σ) controlled by the ferric uptake regulator, Fur, (37) including *pvdS* and *femI* were downregulated in NO treated cells (dispersed cells and biofilms) compared to untreated cells. Genes for pyoverdine synthesis appeared to be downregulated, such as *pvdA* (34), which expression was 7 fold lower in NO treated biofilms compared to untreated biofilms and 6 fold lower in dispersed cells compared to planktonic cells. *tonB1*, an essential component of the siderophore-mediated iron uptake system (38) was also decreased by 11 fold in NO treated biofilms compared to untreated biofilms. Iron receptors, including *fpvA*, *optI* and *hasR* exhibited lower expression levels in NO treated biofilms compared to untreated biofilms. Among these genes, the expression level of *fpvA* was 8 fold lower in NO treated biofilms compared to untreated biofilms. Moreover, the expression of *pvdQ* was nine times lower compared to untreated biofilms and *pchA-D*, *pchR* (39), involved in pyochelin biosynthesis, were also downregulated in NO treated biofilms. In addition, the expression levels of phenazine biosynthesis genes, which encode redox-active pigments involved in QS, virulence and iron acquisition (40), *phzA1*, *phzB2*, *phzC1*, *phzC2*, *phzD1*, *phzD2*, *phzE1*, *phzE2*, *phzF1*, *phzF2*, *phzG1* and *phzG2* were decreased at least 4 fold in NO treated biofilms. *phzA1*and *phzB1*were

147 downregulated in dispersed cells compared to planktonic cells. Therefore, the data suggest that
148 the pyoverdine and pyochelin synthesis genes, as well as iron acquisition related genes, were
149 generally reduced after NO treatment. In contrast, *bfrB*, encoding a bacterioferritin, which is an
150 important iron storage protein in *P. aeruginosa* (41), was highly upregulated in NO treated
151 remaining biofilms (72 fold) and NO dispersed cells (77 fold) compared to untreated cells.
152
153 Since the expression levels of most iron acquisition related genes (e.g., *pvdA*, *pvdS* and *fpvA*)
154 were downregulated after exposure to 100 μM NO donor SP-NO, the effect of NO on pyoverdine
155 production was further investigated. Pyoverdine production was reduced 25% after adding 100
156 μM SP-NO for 15 min (Fig. 1). Similar results were observed after 30 min exposure. Overall,
157 these data suggest a link between the repression of iron acquisition related genes induced by NO
158 and the regulation of biofilm dispersal.
159
160 **Iron overrides NO induced dispersal independent of NO scavenging pathways**
161 To determine whether NO induces biofilm dispersal through inhibition of iron uptake systems,
162 the impact of the addition of exogenous iron on NO induced dispersal was explored. Biofilms
163 were first treated with different concentrations of ferrous iron and NO simultaneously for 30 min.
164 After treatment with 100 μM of the NO donor SP-NO alone for 30 min, about 90% of biofilms
165 were dispersed (Fig. 2A). In contrast, when ferrous iron was added to biofilms at the same time
166 as NO, the dispersal response appeared to be inhibited in an iron dose dependent manner (Fig.
167 2A), with only 40% of the biofilms dispersed after 30 min in the presence of 100 μM $FeSO_4$.
168 These data also show that in the presence of iron alone, the biofilm biomass increased compared
169 to biofilms that had not received iron. This suggested two possibilities, (i) that iron may interfere
170 with NO sensing and the induction of dispersal, or (ii) that iron may affect the dispersal process

171 further downstream in the regulatory cascade. To address this, biofilms were first exposed to NO
172 alone for 15 min, which is sufficient for the induction of dispersal as shown in our previous work
173 (19), before iron was added to the cultures. Even when iron was added 15 min after NO, the
174 biofilm biomass was found to increase in the presence of iron, with 100 μM $FeSO_4$ resulting in a
175 4.9 and 4.7 fold increase after 15 or 30 min, respectively (Fig. 2B). This suggested that the effect
176 of iron on dispersal was not dependent on its presence during NO release, NO sensing or the
177 onset of dispersal. Similar increases in biofilm biomass were found after cells were exposed to
178 100 μM ferric iron $FeCl_3$ (Fig. 2B), indicating that this phenotype was not dependent on the iron
179 oxidation state (i.e. ferrous Fe^{2+} vs. ferric Fe^{3+}).
180
181 Iron is known to have a direct effect on free NO via redox reactions (21). To investigate whether
182 iron directly scavenges NO and consequently inhibits NO induced dispersal, NO specific
183 electrodes were used to measure the amount of free NO released from NO donor SP-NO in the
184 presence of $FeCl_3$, $FeSO_4$ or the NO scavenger PTIO (Fig. 2C). In the absence of any iron or
185 scavenger, the amount of NO liberated from SP-NO reached a steady state of approximately 4
186 μM within 15 min. The subsequent addition of 100 μM $FeSO_4$ caused a dramatic reduction of
187 free NO within 5 min after which time, the amount of NO increased to similar levels as the
188 control (no iron). In contrast, 100 μM $FeCl_3$ had no effect on the amount of NO released.
189 Exposure of *P. aeruginosa* to either form of iron resulted in increased biofilm biomass (Fig. 2D).
190 To further confirm that the inhibitory effect of iron on dispersal was not related to scavenging of
191 NO by iron, the NO scavenger PTIO was added to the SP-NO solution instead of iron. The
192 addition of 200 μM PTIO caused a dramatic reduction of free NO that lasted for the duration of
193 the experiment (Fig. 2C). PTIO was also added into cultures that had been dispersed by NO for
194 15 min. After 15 and 30 min exposure to PTIO, the biofilms remained dispersed (Fig. 2D). Thus,

195 in contrast to iron addition, PTIO mediated scavenging of NO did not lead to hyperbiofilm
196 formation. Ferrous iron showed a transient reduction in NO, inhibited biofilm dispersal and
197 induced hyper biofilm formation. Ferric iron did not scavenge NO, but inhibited dispersal as well
198 as inducing hyperbiofilm formation. These results suggest that iron overrides the NO induced
199 dispersal response and this effect is independent of NO scavenging. Subsequent experiments
200 were performed with $FeCl_3$ to avoid issues of the short-term loss of NO after adding $FeSO_4$.
201
202 **Biofilms formed in the presence of iron can be dispersed by NO**
203 Since iron did not appear to inhibit dispersal by directly scavenging NO, an alternative
204 possibility is that it may induce a cellular response that shuts down the ability of *P. aeruginosa*
205 cells to disperse in the presence of NO signals. To explore whether the presence of iron can fully
206 abolish NO induced dispersal or that iron and NO compete through the same regulatory pathway,
207 the order of NO and iron addition was switched. *P. aeruginosa* cells were first exposed to iron for
208 30 min before NO treatment for 15-60 min. Biofilms that had not been treated with iron and
209 biofilms that had been treated with 100 µM $FeCl_3$ for 30 min were dispersed by 81% and 82%,
210 respectively, after subsequent exposure to 100 µM SP-NO for 15 min (Fig. 3A). However, 60
211 min after the addition of NO, biofilms not pretreated with iron remained dispersed, while
212 biofilms pretreated with 100 µM $FeCl_3$ increased in biomass 3.9 fold compared to untreated
213 control biofilms. Similar results were obtained when biofilms were pretreated with $FeSO_4$ before
214 NO (Fig. 3B). To better understand these changes in biofilm biomass, similar experiments were
215 performed with shorter time frames. Surprisingly, biofilms pretreated with iron dispersed in the
216 first 15 min of exposure to NO before increasing biomass again. The biomass of biofilms
217 pretreated with iron and subsequent exposed to NO decreased by 45%, 58% and 66% compared
218 to iron pretreated biofilms after 5, 10 and 15 min, respectively. However, after 20 min the

biomass of these biofilms started to increase and after 60 min, reached 2.7 fold of untreated control biofilms. Overall, these results reveal that biofilms that are formed in the presence of iron can still be dispersed by NO, and that the biofilm biomass increases rapidly in the presence of iron.

Iron induces rapid (re-)attachment of NO induced dispersed cells and planktonic cells

In the above multiwell plate batch cultures biofilm dispersal assay, iron was added directly into 6 h bacterial cultures that were composed of biofilms and planktonic cells. It is possible that the increase in biofilm biomass observed in those experiments was due to increased growth of the remaining biofilm or was due to the rapid attachment of the suspended cells containing both dispersed and planktonic cells. First, to determine if iron supplementation increases cell growth and if NO treatment inhibits cell growth, colony forming units (CFUs) of *P. aeruginosa* cells that had been previously incubated in multiwell plate batch cultures for 6 h and subsequently exposed to iron and NO were enumerated. After adding iron for 15, 30 or 60 min, the CFUs did not increase (Fig. 4A), suggesting that iron does not promote cell growth under these conditions. Further, after treatment with NO for 15, 30 or 60 min, CFUs did not decrease, indicating that NO was non-toxic at the concentrations used in this study (Fig. 4A).

Second, the planktonic phase and the biofilm phase of pregrown bacterial cultures were separated after NO treatment and before adding iron, in order to distinguish a potential effect of iron on attachment of suspended cells from increased growth of already attached biomass. Culture supernatants were transferred to another well and fresh medium added to the remaining biofilms. Iron was then added into the wells containing only the suspended cells or the wells containing only the non-dispersed biofilms. The data show that, in the absence of NO, the

suspended cells attached rapidly when put in contact with a clean, uncolonized surface. Further, the attached biomass increased by 2.4, 3.2 and 6.2 fold after adding 100 μM $FeCl_3$ for 15, 30 and 60 min, respectively (Fig. 4B). In the presence of NO (without iron), the suspended cells showed very little attached biomass after being transferred to, and incubated in, a clean, new plate for 15 and 30 min. The biomass of NO treated, suspended cells increased by 9.1, 8.8 and 4.1 fold in the presence of $FeCl_3$ for 15, 30 and 60 min, respectively (Fig. 4B). Those results suggest that NO prevents rapid attachment of planktonic cells, while iron induces rapid attachment regardless of the presence of NO. In contrast, the dispersing effect of NO lasted for 60 min and the presence of iron did not enhance the biomass of NO treated remaining biofilms (Fig. 4C). Overall, the above results indicate that iron increases the biofilm biomass of NO treated biofilms mainly by promoting the rapid attachment of planktonic cells or reattachment of dispersed cells rather than through accelerating the growth of cells already within the remaining biofilms.

We have repeated the experiment shown in Fig. 4B and C using a clinical isolate *P. aeruginosa* PA_D25 (42), which was collected from a patient with ventilator-associated pneumonia. As shown in the supplemental material Fig. S1, although the clinical strain was a poor biofilm former overall, the biofilms were dispersed by NO and iron increased the biofilm biomass of NO treated biofilms. As observed for PAO1, this effect of increased biomass was mainly through the promotion of rapid attachment of planktonic cells or reattachment of dispersed cells (A) rather than through accelerating the growth of the remaining biofilms (B).

psl is required for iron induced fast attachment of planktonic cells

Psl, Pel and alginate are the three main exopolysaccharides involved in biofilm development and antibiotic resistance in *P. aeruginosa*. High levels of iron (50 and 100 μM) have been recently

267 reported to promote biofilm formation in *P. aeruginosa* by increasing the production of Psl (24).
268 To determine if iron facilitated rapid attachment of planktonic cells through inducing the
269 biosynthesis of those exopolysaccharides, *P. aeruginosa* polysaccharide-deficient mutants
270 including Δ*pel*, Δ*psl* and Δ*alg* were tested (Fig. 5A). *P. aeruginosa* wild type and Δ*pel* showed
271 approximately 1.3 and 2.0 fold increases, respectively, in biofilm biomass after treatment with
272 100 μM $FeCl_3$. The Δ*alg* mutant did not grow well in the current experimental conditions and its
273 OD_{600} remained at the detection limit, 0.01, after 6 h incubation (Fig. 5B). In contrast, the Δ*psl*
274 mutant grew well in the planktonic phase (Fig. 5B), but failed to form biofilms (Fig. 5A),
275 suggesting that Psl is important for biofilm formation in these experimental conditions. Further,
276 no attachment of the Δ*psl* planktonic cells was found in the presence of 100 μM $FeCl_3$ (Fig. 5A).
277 The results presented above suggest that Psl is required for the iron induced rapid attachment of
278 planktonic cells, although it remains to be determined if iron induced the production of Psl to
279 enhance attachment.
280
281 To investigate whether iron and NO influence biofilm development through controlling Psl
282 production or other mechanisms, Psl of *P. aeruginosa* biofilms before and after iron or NO
283 treatment were quantified by using a Psl-specific fluorescent stain, TRITC-HHA (43), and
284 microscopy analysis. After adding 100 μM $FeCl_3$ for 30 min, biofilm biomass increased by 1.9
285 fold (Fig. 6B) and Psl production increased by 1.2 fold (Fig. 6C). These results confirmed that
286 100 μM $FeCl_3$ promoted Psl production and induced cell attachment. In contrast, treatment with
287 100 μM NO donor for 30 min resulted in 71% biofilm dispersal (Fig. 6B) but only lead to a
288 slight reduction (14%) in the Psl bound TRITC-HHA fluorescent signals (Fig. 6C). Confocal
289 images showed that most Psl still remained on the surface of the microtitire wells after NO
290 treatment (Fig. 6A). Overall these results suggest that iron induces attachment by promoting the

291 production of Psl, while NO may not only induce dispersal by degrading Psl, but also by altering
292 another mechanism of *P. aeruginosa* attachment (discussed below).
293
294 **Iron-limited biofilms become more sensitive to NO**
295 The data above show that NO induces a concomitant decrease in pyoverdine production at the
296 same time as inducing dispersal of biofilm cells and that freshly dispersed cells can rapidly
297 reattach in the presence of iron. These observations suggest that depleting iron by the use of a
298 chelator could potentially inhibit the reattachment of dispersal cells and enhance the dispersal
299 effect of NO. To investigate whether iron depletion enhanced or interfered with NO induced
300 dispersal, biofilms were treated with NO in the presence or absence of the iron chelator, 2,2'-
301 Bipyridine (Bipy). The iron chelator alone had no significant impact on biofilm biomass (Fig. 7).
302 In these experiments, the NO donor SP-NO was used at 50 μM, a lower concentration that results
303 in the rapid depletion of NO and reattachment of biofilms, with the biomass of SP-NO treated
304 biofilms increasing back to 53% of the untreated biofilm biomass after 30 min and to 85% after
305 60 min. In contrast, when 50 μM NO donor was added together with 200 μM Bipy, 74% of the
306 biofilms were removed after 30 min, and this effect was prolonged after 60 min (Fig. 7). Taken
307 together, these results indicate that iron depletion can potentiate NO induced biofilm dispersal.
308
309 **DISCUSSION**
310 **The interplay between iron and NO in controlling biofilm development**
311 NO and iron are two important environmental cues that control biofilm formation. This study has
312 explored the interplay between these signaling molecules in the regulation of *P. aeruginosa*
313 biofilms and found that: (i) NO induces a decrease in several iron acquisition related genes as
314 well as a decrease in pyoverdine production, (ii) iron overrides NO induced biofilm dispersal

315 through promoting a rapid reattachment of dispersed cells, which involves Psl production and
316 (iii) combined treatments of NO with an iron chelator can enhance biofilm dispersal.
317
318 A link between NO mediated dispersal and reduced expression of iron acquisition related genes
319 has been observed before (7, 27). However, in these studies, the NO donor that was used, sodium
320 nitroprusside, contains an iron moiety and has been previously found to release iron ions, which
321 could have a potential impact on pyoverdine production independent of NO (44). Here, the
322 effects were observed using a NONOate NO donor that does not contain any iron. Bacteria in
323 biofilms are usually associated with a physiology indicative of iron limitation, which is likely
324 due to the high cell density and limited availability for this nutrient. The potential coregulation of
325 dispersal with reduced expression of iron acquisition is interesting since dispersal cells are likely
326 to encounter increased levels of iron once they are treated with NO. It is unclear why *bfrB* is
327 highly expressed after NO treatment. One possibility is that NO induces *bfrB* through Fur since
328 *bfrB* is predicted to be regulated by Fur (45, 46). A recent study revealed that accumulation of
329 iron-binding by BfrB induces acute iron deprivation in the cytoplasmic space as well as the de-
330 repression of iron acquisition genes (41). However, in our study, NO was found to induce *bfrB*
331 expression, while iron acquisition genes were repressed. Our results may differ from the previous
332 study in that while 15 min after NO treatment *bfrB* is highly induced, binding of intracellular iron
333 by BfrB may not be significant yet thus maintaining repression of iron acquisition genes. We also
334 tested a *bfrB* mutant and observed that it still dispersed to the same extent as the *P. aeruginosa*
335 PAO1 wild type (data not shown), suggesting that BfrB does not contribute to biofilm dispersal.
336 Compared to the strong repression of several pyoverdine synthesis genes, the reduction of
337 pyoverdine by NO was much less. It is possible that NO does not affect the existing pool of
338 pyoverdine at 6 h, but rather inhibits transcriptional control and hence, changes in pyoverdine

levels may lag well behind the repression of gene expression. Although the exact mechanism of NO regulating *pvd* genes and pyoverdine production remains to be elucidated, this effect is likely to involve c-di-GMP. Indeed, NO is known to induce a reduction in c-di-GMP levels (7), and in turn decreased c-di-GMP has been found to abolish pyoverdine production (29). This study has shown that addition of iron, which is also known to reduce *pvd* expression, causes enhanced attachment of bacteria rather than promote dispersal, which suggests that the decrease of *pvd* induced by NO is unlikely to be the cause of the downstream dispersal response, but may rather be a parallel, unrelated regulatory effect.

NO and iron induced signaling pathways appear to be independent but converge on the downstream attachment effectors

The results shown here suggest that NO and iron do not operate via overlapping signaling pathways, as iron does not strictly inhibit the dispersal response and NO dispersed cells can still attach in response to iron. However, the response to both iron and NO appears to involve the exopolysaccharide Psl. A recent study has reported that high iron (50 and 100 μM $FeCl_3$) promoted Psl production and thus induced biofilm formation (24), which correlates with the data presented here. Fast attachment responses involving Psl polysaccharides have already been observed in *P. aeruginosa*, including in response to surfactant stress. For example, SDS surfactants have been shown to induce aggregation and attachment of suspended cells within 45 min, via c-di-GMP and Psl (47). Interestingly, while the addition of iron can induce attachment in a Psl-dependent manner, the results presented here suggest that chelation of iron alone in the absence of NO treatment does not promote dispersal (Fig. 7). In *P. aeruginosa*, the intracellular concentration of c-di-GMP is known to regulate Psl production at both the transcriptional and translational levels (44). While it has been shown, as indicated above, that NO exposure can

result in reduced c-di-GMP concentrations, it remains unclear if there is a direct link between NO and Psl production. In contrast the effect of iron on Psl does not appear to involve c-di-GMP as it was previously found that the addition of iron had no impact on c-di-GMP levels (24). Another important regulatory pathway that drives the transition between biofilms and planktonic cells in *P. aeruginosa* is the acylated homoserine lactone (AHL)-based QS system. High levels of iron have been suggested to promote Psl production through the repression of the QS controlled genes *rhlAB*, *rhlI* and *rhlR*, thus reducing the synthesis of rhamnolipids as well as inhibiting *amrZ*, which encodes a transcriptional factor that inhibits transcription of the *psl* operon (48-50). Therefore, it is possible that iron may control Psl through modulating QS. A potential link between iron, biofilms and QS could be explored in future studies by investigating QS mutants using the iron induced fast attachment assay reported here.

Moreover, in this study, NO had little impact on Psl levels and most Psl remained attached to the surface after NO treatment, despite dispersal of most of the biofilm (Fig. 6). Intriguingly, the dispersal response to NO has been found to require the periplasmic protease LapG (17), which can cleave the protein adhesin CdrA off the cell surface (51). CdrA is known to either cross-link Psl polysaccharide polymers and/or tethers the cells to the Psl polysaccharides (3). Taken together, these observations suggest that addition of NO may cause the cleavage of CdrA off the cell surface through c-di-GMP and LapG and therefore break the link between biofilm cells and Psl, finally resulting in the release and dispersal of bacteria.

Combined treatments of NO and iron chelators may improve biofilm control

In natural environments, bacteria can encounter varying levels of available iron. For example, iron is present in wastewater at an average of 9 μM (52), while in the cystic fibrosis (CF) lung,

387 the iron concentration varies from 2 to 130 μM (53-57). Therefore, the presence of iron could
388 significantly impact the efficacy of NO when applied in industrial and clinical settings. A range
389 of iron chelating compounds for biofilm control has been studied previously (58-60). In this
390 work, the iron chelator Bipy was used in combination with NO and it was observed that NO
391 treated biofilms were more sensitive to iron limitation and dispersed to a greater degree than
392 biofilms in the absence of iron chelator. Further, previous studies have also reported that the
393 combination of NO, iron chelator and tobramycin efficiently reduces the survival of *P.*
394 *aeruginosa* dispersed cells (27). Therefore, simultaneous treatments of biofilms with NO, an iron
395 chelator and biocides could be a powerful way to remove and kill biofilms in iron-rich
396 environments.
397
398 In summary, this study has shown that two important environmental signals, iron and NO,
399 control biofilm development in opposing ways through different pathways, which appear to be
400 both linked to the polysaccharide Psl. Furthermore, iron chelator and NO were found to have a
401 synergetic effect in dispersing biofilms, which suggests new ways for improving the use of NO
402 in biofilm control strategies.
403
404 **MATERIALS AND METHODS**
405 **Bacteria and growth conditions.** The *P. aeruginosa* PAO1 wild type (WT) strain (61), as well
406 as *P. aeruginosa* mutant strains, including Δ*pel* (isogenic *pelF* deletion mutant) (62), Δ*psl*
407 (isogenic *pslA* deletion mutant) (62) and Δ*alg* (isogenic *alg8* deletion mutant) (62) were used
408 here. Bacteria were routinely grown in Luria-Bertani (LB) Miller broth (BD Difco) overnight at
409 37°C with shaking at 200 rpm for 16 h to prepare cells for experiments.
410 **Biofilm dispersal and attachment assays.** Biofilms were grown and dispersed as previously

411 described (63) with some modifications as explained in our previous work (19). For bacterial
412 attachment assays, *P. aeruginosa* cultures were grown in multiwell plates using the same
413 experimental settings as for biofilm dispersal assays. After 6 h, $FeCl_3$ (Sigma-Aldrich), $FeSO_4$
414 (Sigma-Aldrich) or the NO scavenging compound 2-(4-carboxyphenyl)-4,4,5,5-
415 tetramethylimidazoline-1-oxyl-3-oxide potassium salt (Carboxy-PTIO potassium salt) (Sigma-
416 Aldrich) was added into the cultures that had received or not NO treatment. The final
417 concentration of iron salts was 100 µM and the concentration of PTIO was 200 µM. The plates
418 were incubated for a further period from 5 min to 60 min. After the final incubation, biofilm
419 biomass was quantified using CV staining as described before (19).
420 **Pyoverdine quantification.** Production of pyoverdine in supernatants of *P. aeruginosa* cultures
421 was quantified by measuring the natural fluorescence of the culture (excitation wavelength, 400
422 nm; emission wavelength, 450 nm; Infinite Pro2000 microplate reader, Tecan) (64) and the
423 pyoverdine production level was normalized to the OD_{600} values for each well.
424 **Amperometric measurements of NO.** The concentration of NO liberated from NO donor was
425 measured amperometrically by using a TBR1025 Free Radical Analyzer (World Precision
426 Instruments) equipped with a NO specific ISO-NOP 2 mm electrode, with a detection range from
427 1 nM to 100 µM and calibrated by using MAHMA NONOate (Cayman Chemical) as the NO
428 donor. After allowing the amperometric signal to stabilize in M9 salts, 100 µM SP-NO was
429 added. Then after 15 min, 100 µM $FeSO_4$, 100 µM $FeCl_3$ or 200 µM PTIO was added. These
430 experiments were repeated at least 3 times.
431 **Biofilm, Psl staining and microscopy analysis.** *P. aeruginosa* wild-type biofilms grown in
432 multiwell plate batch cultures for 6 h and subsequently left untreated or treated with 100 µM
433 $FeCl_3$ or 100 µM NO donor SP-NO for 30 min were rinsed once with PBS before being stained
434 with 50 µg/mL tetramethyl rhodamine isothiocyanate (TRITC)-labeled hippeastrum hybrid lectin

(amaryllis) (HHA) (EY Labs, Inc.) for 1 h, as previously described (43). Biofilms were subsequently rinsed twice with PBS before being stained with Syto9 (Molecular Probes, Inc.). Briefly, 1.5 µL Syto9 was diluted in 1 mL of PBS, and then 0.5 mL of this solution was added into each well and incubated at room temperature in the dark for 15 min. Images of untreated and iron or NO treated biofilms were acquired through the culture well's bottom surface by using inverted confocal laser scanning microscopy (CLSM) (Carl Zeiss Microscopy; LSM 780). Biofilm quantification was performed using the IMARIS software package (Bitplane AG).

Statistical analysis. Multivariate analyses were performed using one-way ANOVA and two-way ANOVA, followed by Sidak post-test for individual comparisons. Asterisks in figures indicate statistically significant differences compared to untreated control samples or between different samples (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

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456

457 The authors declare no conflict of interest.

458

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636 **FIGURE LEGENDS**

637 **FIG 1. NO inhibits pyoverdine production in *P. aeruginosa*.** Biofilms were grown in multiwell
638 plate batch cultures for 6 h and treated with 100 µM NO donor SP-NO for 15 or 30 min. Relative
639 fluorescence units (RFU) of pyoverdine were calculated by normalizing the pyoverdine
640 fluorescence to the OD_{600} measurement of the culture density. Error bars indicate standard
641 deviation (n = 8). Asterisks indicate statistically significant differences compared to untreated
642 control samples ($P \leq 0.05$).

643

644 **FIG 2. Iron overrides NO induced biofilm dispersal.** (A) *P. aeruginosa* biofilms were grown
645 in multiwell plate batch cultures for 6 h and treated with 100 µM NO donor SP-NO and different
646 concentrations of $FeSO_4$ simultaneously for 30 min before quantifying the biofilm biomass by
647 CV staining. Each image represents the stained biofilms. (B & D) Biofilms grown in multiwell
648 plate batch cultures for 6 h and treated with 100 µM SP-NO for 15 min were subsequently
649 treated with 100 µM $FeCl_3$ or $FeSO_4$ (B) or 200 µM PTIO (D) for 15 or 30 min before CV
650 staining. Error bars indicate standard deviation (n = 6). Asterisks indicate statistically significant
651 differences compared to untreated control samples ($P \leq 0.05$). (C) Effect of $FeCl_3$, $FeSO_4$ and
652 PTIO on NO release. Levels of free NO were monitored amperometrically in a solution to which
653 100 µM NO donor SP-NO was added at t=0 min. After 15 min, 100 µM $FeCl_3$, 100 µM $FeSO_4$ or
654 200 µM PTIO were added to the solution as indicated by the arrow. The results are representative
655 of at least three independent experiments.

656

657 **FIG 3. Biofilms formed in the presence of iron can be dispersed by NO.** (A) *P. aeruginosa*
658 biofilms were grown in multiwell plate batch cultures for 6 h and pretreated with 100 µM $FeCl_3$
659 for 30 min. Biofilms were then treated with 100 µM SP-NO for 15, 30 or 60 min before CV

staining. (B) Six hour biofilms were pretreated with 100 µM $FeSO_4$ for 30 min. Biofilms were then treated with 100 µM SP-NO for 5 to 60 min before CV staining. Error bars indicate standard deviation (n = 4). Asterisks indicate statistically significant differences compared to untreated control samples ($P \leq 0.05$).

FIG 4. Iron overrides NO induced biofilm dispersal mainly via promoting rapid attachment of suspended cells. (A) The effect of iron and NO on the growth of *P. aeruginosa* was tested. *P. aeruginosa* biofilms were grown in multiwell plate batch cultures for 6 h and subsequently, supernatants containing planktonic cells were collected and transferred to 50 ml tubes. Bacterial cells were incubated in the absence and presence of 100 µM $FeCl_3$ or SP-NO for 15, 30 and 60 min and CFUs were calculated. Error bars indicate standard deviation (n = 9). Asterisks indicate statistically significant differences compared to untreated control samples ($P \leq 0.05$). Further, *P. aeruginosa* biofilms grown in multiwell plate batch cultures for 6 h and subsequently dispersed by 100 µM NO donor for 15 min. Supernatants containing planktonic cells and dispersed cells were then transferred to a new empty multiwell plate and fresh M9 medium was added into the original plate with the remaining biofilms. The two plates containing either the supernatants (B) or the remaining biofilms (C) were then incubated as before and treated or not with 100 µM $FeCl_3$ for 15, 30 and 60 min before CV staining. Error bars indicate standard deviation (n = 6). Asterisks indicate statistically significant differences compared to untreated control samples or between different samples ($P \leq 0.05$).

FIG 5. Iron does not promote rapid attachment of planktonic cells of *P. aeruginosa* Δ*psl* mutant. Biofilms of *P. aeruginosa* wild type (WT) and mutant strains Δ*pel* (isogenic *pelF* deletion mutant), Δ*psl* (isogenic *pslA* deletion mutant) and Δ*alg* (isogenic *alg8* deletion mutant)

were grown in multiwell plate batch cultures for 6 h and treated with 100 µM $FeCl_3$ for 30 min before CV staining of the biofilm biomass (A) and OD_{600} measurement of the planktonic biomass (B). Each image represents the stained biofilms. Error bars indicate standard deviation (n = 3). Asterisks indicate statistically significant differences compared to untreated control samples ($P \leq 0.05$).

FIG 6. Psl production increased in iron treated biofilms and slightly decreased after NO treatment. *P. aeruginosa* biofilms grown in multiwell plate batch cultures for 6 h and subsequently treated with or without 100 µM $FeCl_3$ or 100 µM NO donor SP-NO for 30 min were stained with Syto9 and TRITC-HHA and analyzed by using confocal microscopy (A). Biofilm cells appear green and Psl polysaccharides appear red. The main central images show horizontal optical sections (x-y) of the biofilms, and the side and top panels show vertical optical sections (x-z and y-z, respectively). Scale bars are 10 µm. Image analysis was used to quantify biofilm (B) and Psl (C) signals from reconstructed 3 dimensional image stacks. Error bars indicate standard deviation (n = 12). Asterisks indicate statistically significant differences compared to untreated control samples ($P \leq 0.05$).

FIG 7. Iron-limited biofilms become more sensitive to NO. Biofilms grown in multiwell plate batch cultures for 6 h were treated with 50 µM SP-NO, 200 µM Bipy or the combination of both for 15, 30 and 60 min. Biofilm biomass was quantified by CV staining. Error bars indicate standard deviation (n = 6). Asterisks indicate statistically significant differences compared to untreated control samples or between different samples ($P \leq 0.05$).