Mechanisms of nitric oxide-mediated biofilm dispersal in *Pseudomonas aeruginosa*

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NANYANG
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**MECHANISMS OF NITRIC OXIDE-MEDIATED
BIOFILM DISPERSAL IN *PSEUDOMONAS*
*AERUGINOSA***

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**SINGAPORE CENTRE FOR ENVIRONMENTAL LIFE
SCIENCES ENGINEERING (SCELSE)**

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BIOFILM DISPERSAL IN *PSEUDOMONAS*
*AERUGINOSA***

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A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of
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Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Abstract

The biological signal molecule nitric oxide (NO) was found to induce biofilm dispersal across a range of bacterial species including *Pseudomonas aeruginosa*, which led to its consideration for therapeutic strategies to treat biofilms and biofilm-related infections. However, despite the clear link between NO and the bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), a secondary messenger molecule in bacteria that controls the transition between planktonic cells and biofilms, the direct NO sensor that can transmit NO signal into intracellular changes and c-di-GMP levels has not been fully elucidated in *P. aeruginosa*. It has also been observed that biofilms are often not completely dispersed after exposure to NO and multiple-doses of NO do not improve biofilm dispersal. To better understand those phenomena, to identify putative NO binding receptors and to elucidate the molecular pathway of NO induced dispersal, this study has successfully created transcriptomic profiles of both messenger RNA and small, non-coding RNA in *P. aeruginosa* untreated planktonic cells and biofilms as well as the cells remain within biofilms after NO treatment and NO dispersed

cells. Based on the RNA-Seq data, this study has highlighted several new targets that may be involved in NO induced dispersal. Moreover, this research has linked two important environmental signals (iron and NO) with their roles in biofilm development. Furthermore, it was found that the transcriptional factor FhpR can sense NO to trigger production of the NO binding protein, flavohemoglobin Fhp, which was linked to incomplete biofilm dispersal. Overall, work presented in this thesis has addressed the mechanisms of NO induced biofilm dispersal in *P. aeruginosa* and offered new perspectives for improving the use of NO in biofilm control strategies via using imidazole to inhibit Fhp and using iron chelators to control iron concentrations in the environment.

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Chapter One: Literature Review

1.1 Introduction

The present thesis addressed the mechanisms of Nitric oxide (NO) induced biofilm dispersal in *Pseudomonas aeruginosa* as well as potential problems and solutions when use NO in biofilm control strategies. The exogenous addition of NO has been shown to induce dispersal of biofilms formed by a broad range of microorganisms via modulating bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP). However, despite the clear link between NO and the c-di-GMP signaling pathway, there are several aspects of the regulatory mechanisms that are not well understood. For example, the direct NO sensor that can transmit NO signal to c-di-GMP has not been fully elucidated in *P. aeruginosa*. Therefore, a global, non-targeted approach investigating the change in transcriptome between NO treated and untreated cells is necessary. A RNA sequencing study and a microarray study that compared the NO treated and untreated biofilms have been previous reported (1). However, the profiles of both the cells remaining within the biofilms after NO treatment and the NO dispersed cells were not compared in the same system in those studies. Further, a complete profile of small non-coding RNAs (sRNAs), which may be involved in the regulation of biofilm dispersal, has not been built. Moreover, the possible interaction of NO with other signaling systems, for example, iron, in biofilm development has not been investigated yet. Understanding those questions as mentioned above has implication for being aware of challenges when using NO as biofilm control strategy in both industrial and clinical settings. For example, the presence of iron may have a significant impact on biofilm control and

repeated exposure of biofilms to NO does not improve biofilm dispersal. The underlying mechanisms and solutions regarding those problems are also discussed in this thesis.

1.2 *P. aeruginosa* and biofilms

P. aeruginosa is a ubiquitous and versatile soil and water Gram-negative bacterium, as well as an opportunistic pathogen that produces many virulence factors that are important for colonization, survival, cytotoxicity and evasion of the innate host immune system (2, 3). As a human opportunistic pathogen, *P. aeruginosa* is responsible for severe nosocomial infections in immunocompromised patients and causes life-threatening, chronic lung infections in cystic fibrosis (CF) patients (4). *P. aeruginosa* has been reported to be one of the most common hospital pathogens and is the second most common pathogen isolated from patients with ventilator-associated pneumonia in US (5). One of the main reasons for the success of *P. aeruginosa* as a human pathogen is its ability to form biofilms (6).

Bacterial biofilms are matrix-encased aggregates of microorganisms in which cells attach to a surface or adhere to each other (7) and are essential to the ecology and biology of bacteria (8, 9). In the form of biofilms, bacteria become much more resistant to biocides, antibiotics and immune systems compared to their free-living planktonic cells (10). Biofilm cells show genetic and physiological heterogeneity that are caused by microscale chemical gradients, adaptation to environmental conditions, stochastic gene expression and genotypic variation (11). Such heterogeneity of biofilms constitutes an important strategy to defend themselves against antimicrobials (12). A broad

range of pathogens can cause life-threatening infections when they form biofilms on tissues, including in the lungs, nose, urinary tract, ears, heart, oral cavity and wounds as well as on abiotic surfaces such as contact lenses and other indwelling biomedical devices (13, 14). For example, *P. aeruginosa*, which is one of the best studied organisms for bacterial biofilm formation, is responsible for many infections including chronic lung infections in CF patients and biofilm formation in chronic wounds such as those of severe burn victims (15).

One of the main matrix components in *P. aeruginosa* biofilms are exopolysaccharides. Three main types of exopolysaccharides in *P. aeruginosa* biofilm matrix are PSL, which is composed of repeating pentamer consisting of D-mannose, L-rhamnose and D-glucose (16), PEL, a glucose-rich polysaccharide (17) and alginate, which is composed of 1,4-linked β -D-mannuronic acid and α -L-guluronic acid (18). The PEL polysaccharide contributes to biofilm development post-attachment (19) while PSL is anchored on the surface in a helical pattern during attachment and promotes cell-cell interactions as well as assembly of the matrix (20). The production of both PEL and PSL are under control of c-di-GMP, which is a secondary messenger molecule in bacteria that controls the transition between planktonic cells and biofilms through stimulating exopolysaccharide synthesis, flagella and pili based motility (21, 22). C-di-GMP has been reported to positively regulate PEL biosynthesis at the post-translation level and also regulates the production of PSL at both transcriptional and translation levels (23).

Bacterial biofilms develop through several stages that include initial reversible attachment, irreversible attachment, maturation and dispersion (24). The final

stage, dispersal, is an active response, different from cell sloughing and erosion-induced passive dispersal (25, 26). Biofilm dispersal can be triggered by a variety of environmental cues including low levels of NO (26, 27), oxygen (O_2) depletion (28, 29), changes in temperature (30), changes in iron levels and nutrient availability (31-34). It is therefore of great clinical interest to study biofilm dispersal with the aim of inhibiting biofilm formation or promoting biofilm dispersal. In particular, the role of NO in regulating biofilm dispersal offers a unique opportunity for biofilm control as it is highly conserved across bacterial species (35).

1.3 Role of NO in *P. aeruginosa* biofilm dispersal

1.3.1 NO responsive networks in *P. aeruginosa*

NO is a hydrophobic uncharged gas molecule and a highly reactive free radical with one unpaired electron (36). In nature, bacteria may encounter NO from eukaryotes that release it in response to a bacterial infection (37, 38), or can produce NO endogenously, for example as a free intermediate in the denitrification pathway that is generated through nitrite (NO_2^-) reduction (39, 40). Denitrifying organisms including *P. aeruginosa* are able to reduce nitrate (NO_3^-) to nitrogen (N_2) via a series of sequential reactions with NO_2^- , NO and nitrous oxide (N_2O) as intermediates, and involving the enzymes nitrate reductase (NAR), NO_2^- reductase (NIR), NO reductase (NOR) and N_2O reductase (NOS) (41).

At high concentrations (micromolar to millimolar range), NO may have severe damaging effects to bacterial cells, and as a result many bacteria have evolved defense mechanisms to eliminate NO through various detoxification pathways

(36). Under anaerobic conditions, *P. aeruginosa* can detoxify NO by using a NO reductase, NOR, encoded by *norB* and *norC*, that reductively metabolize NO to N₂O (42). In *P. aeruginosa*, two CRP/FNR-related transcription factors ANR (anaerobic regulator of arginine deiminase and nitrate reductase) and heme sensor-containing DNR (dissimilatory nitrate respiration regulator) control changes in gene expression in response to low oxygen tension, nitrate, nitrite, NO and other N-oxides. Thus, the expression of *norB* and *norC* is induced by DNR upon sensing NO, which creates a self-regulating system to maintain low levels of NO (43). Further, transcription of *dnr* is under control of ANR (44), which can itself be inactivated by NO (45). Additionally, two Fe(II)-containing dioxygenases, HmgA and Hpd, have been found to scavenge NO via an ANR-dependent derepression (45). Finally, KatA, a major catalase whose activity is partly dependent on ANR and requires a basal level of NO, was found to bind and scavenge NO under anaerobic conditions (46). Under aerobic conditions, NO is detoxified by a flavohemoglobin Fhp with NO dioxygenase (NOD) activity (42, 47), which expression is regulated by transcriptional factor FhpR, homologous to the non-heme iron cofactor protein NorR that is capable of sensing NO in *Escherichia coli* (47, 48). Those two genes are adjacent to each other and share a bidirectional promoter region, which is independent of DNR or ANR (47). A schematic diagram of the NO responsive network is shown in Figure 1.1.

1.3.2 Mechanisms of NO induced dispersal

Low, non-toxic concentrations (nanomolar range) of NO cause a transition from the biofilm to the motile planktonic phenotype in *P. aeruginosa* (27). Several studies have revealed that the exogenous addition of NO induces

dispersal of biofilms formed by a broad range of microorganisms including: *E. coli*, *Vibrio cholerae*, *Staphylococcus aureus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Legionella pneumophila*, *Nitrosomonas europaea* and *Neisseria gonorrhoeae* (49-51). In addition, NO was reported to induce dispersal of multi-species biofilms from water systems and to increase their sensitivity to antimicrobial treatments (52). While it is clear that NO can control biofilms formed by a wide range of organisms, the molecular mechanisms that underlie the NO induced biofilm dispersal are poorly defined currently.

Figure 1.1 A schematic diagram of the NO responsive network in *Pseudomonas aeruginosa*. T-shaped lines represent negative control and arrows represent positive control. Dotted lines show links between NO and three NO-responsive regulators: ANR, DNR and FhpR. See text for details. The schematic is adapted from (41).

It has become apparent that c-di-GMP plays an important role in NO induced biofilm dispersal. C-di-GMP is a soluble secondary messenger molecule in bacteria that controls the transition between planktonic cells and biofilms by stimulating exopolysaccharide synthesis, flagella and pili based motility (21, 22). Diguanylate cyclases (DGCs), containing the conserved GGDEF domain, are responsible for synthesizing c-di-GMP from GTP (21, 53). Phosphodiesterases (PDEs), containing the conserved EAL or HD-GYP domains, are responsible for degrading c-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) (21, 54, 55). A direct link between NO, a NO sensor and c-di-GMP was first established in *Shewanella woodyi*, when NO was found to enhance c-di-GMP degradation and to inhibit biofilm formation by binding to H-NOX (heme-nitric oxide/oxygen-binding) domains and regulating the enzymatic activity of a dual-functioning DGC (56). In *E. coli*, NO has been shown to bind to the Per-Amt-Sim (PAS) sensor domain of the Dos protein (57), which contains an EAL domain that possesses c-di-GMP specific PDE activity (58), suggesting that NO may be sensed by the Dos protein and therefore control the c-di-GMP level through modulating the PDE activity of the Dos protein. NO induces the reduction of c-di-GMP in *P. aeruginosa* through the stimulation of enzymatic activity of PDEs including DipA, NbdA and MucR (28, 59, 60). The chemotaxis transducer protein BdlA, which harbours two PAS domains, was found to be involved in NO induced biofilm dispersal (26). BdlA may function as an energy sensor since it does not directly induce c-di-GMP reduction in response to NO (61). The c-di-GMP synthesizing enzyme DGC, GcbA, contributes to NO induced biofilm dispersal by regulating BdlA cleavage (62). Although a direct sensor of NO that mediates c-di-GMP

degradation has not been found in *P. aeruginosa*, a recent study has uncovered a novel NO sensing protein domain, NosP, involved in the regulation of biofilm formation and dispersal via a multi-component phosphorelay system in this bacterium (63). However, the mechanisms by which NO is sensed, as well as how the signal is transmitted to intracellular change, especially gene expressions in *P. aeruginosa* have not been fully elucidated. Therefore, studies on how NO mediates gene expression and the regulation of the planktonic and biofilm modes of growth are necessary to better understand how this molecule mediates biofilm dispersal and improve dispersal strategies to control biofilms.

1.3.3 Gene regulation in NO induced dispersal in *P. aeruginosa*

Microarray based studies reported by Barraud *et al.* showed that physiological concentrations of NO induce changes in the expression of genes involved in anaerobic metabolism, motility, attachment, cell envelope biogenesis, adaptation, protection virulence, c-di-GMP signaling and bacteriophage (26). Gene expression profiles of five-day-old biofilms of *P. aeruginosa* grown in a once-through continuous flow reactor system in the absence and presence of the NO donor sodium nitroprusside (SNP) (1 h) were compared. It was found that several GGDEF and/or EAL domain-containing genes were differentially expressed after NO treatment. Additionally, genes (*pvd*) encoding the enzymes responsible for the biosynthesis of the siderophore pyoverdine were downregulated. In addition, *nirS* and *norC*, involved in the denitrification pathway, were induced in the presence of SNP. Furthermore, type IV pili genes, such as *pilA*, were upregulated upon exposure to exogenously added NO. Conversely, the expression of adhesion genes including *cupB* and *cupC* was inhibited by NO. The work presented by Barraud *et al.* demonstrated that NO

induced dispersal involved a metabolic response rather than a toxic response. However, those studies only compared changes in expression profiles in biofilm cells before and after NO treatment (at the onset of dispersal) and did not characterize the difference in gene expression between the three distinct types of cells (planktonic, dispersed cells and biofilm cells) to explain the dispersal induced by NO. Another limitation of this method is that SNP has an iron centre that may induce biofilm formation (the role of iron in regulating biofilm formation is presented below in section 1.4.2). Therefore, while the results strongly suggest that NO produced by SNP induces dispersal through the specific modification of DGC and PDE encoding genes as well as effectors associated with dispersal, e.g., flagella, it remains a possibility that the secondary effects of SNP, e.g., iron regulation, may confound identification of the NO pathway.

A subsequent study of Chua *et al.* showed that dispersed cells were highly virulent and more sensitive to iron stress compared to planktonic cells (1). In that study, biofilms were grown in microplates with ABTGC medium (ABT minimal medium supplemented with 2 g glucose L⁻¹ and 2 g casamino acids L⁻¹). To eliminate the potential effects of the iron from SNP, cells were dispersed by using SNP and by inducing the expression of a plasmid-encoded YhjH PDE, respectively. Expression of the YhjH PDE resulted in the reduction of intracellular c-di-GMP, which is associated with the regulation of biofilm dispersal. Single-nucleotide resolution transcriptomic analysis was performed to compare the gene expression profiles of four different types of cells, including NO or YhjH-induced dispersed cells, planktonic cells and biofilm cells before treatment. This analysis identified genes involved in type II secretion system

genes (T2SS) (*secD*, *secF*) that were induced in NO or YhjH induced dispersed cells. Subsequent experiments showed that dispersed cells were highly virulent against macrophages and *Caenorhabditis elegans* compared to cells without treatment. Additionally, genes involved in siderophore production, including *pvdS* and *pvdS* regulated genes, e.g. *pvdG*, *pvdH*, *pchB*, *pchC*, *pchD*, *pchE*, *pchG*, *pchF* and *fpvA*, were also downregulated in both SNP and YhjH induced dispersed cells compared to untreated planktonic cells, consistent with the previous data of Barraud *et al.* (1, 26). It was therefore hypothesized that, in addition to modulating c-di-GMP, NO may impact on biofilm development via other pathways such as controlling iron uptake (pyoverdine production) system. However, in the study of Chua *et al.*, the downregulation fold changes of above genes were always higher in SNP induced cells than YhjH induced cells. For instance, *pvdS* was decreased by 277 fold in SNP dispersed cells but was decreased by 54 fold in YhjH dispersed cells, compared to untreated planktonic cells. *fpvA* was decreased by 36 fold in SNP dispersed cells but decreased by 7.3 fold in YhjH induced dispersed cells, compared to untreated planktonic cells. Thus, it is still unclear whether the secondary effects of SNP (iron regulation) could confound identification of the NO pathway.

1.3.4 Roles of small RNA in biofilm development

1.3.4.1 Introduction of small RNA

Bacterial sRNAs generally range from 50 to 250 nucleotides in length and are primarily located in intergenic regions (64, 65). sRNAs function through the post-transcriptional control of gene expression and control a variety of processes including metabolic reactions and stress responses (66). sRNAs work through base-pairing interactions with messenger RNA (mRNAs), in which

case they are referred to as antisense sRNAs, or by binding to and altering the activity of proteins that regulate gene expression (67). Antisense sRNAs can be either *trans*- or *cis*-encoded (68) (Figure 1.2). *Trans*-encoded sRNAs are expressed from distinct loci relative to the transcripts they regulate, whereas *cis*-encoded sRNAs are expressed from the same loci or from the strand opposite their target mRNA and, consequently, are perfectly complementary to at least a portion of their target RNA (69). *Trans*-encoded sRNAs often form limited base-pairing interactions with target mRNAs through the RNA chaperone Hfq (70). RNAs that regulate the activity of a different RNA molecule are *trans*-acting regulators. RNAs that regulate the activity of the same RNA molecule are named as *cis*-acting RNAs (68).

Figure 1.2 Types of small RNAs. Regulatory RNAs can act on other RNA molecules (*trans*-acting) or on themselves (*cis*-acting), and can be expressed from a separate locus (*trans*-encoded) or from the same locus as the target RNA (*cis*-encoded). The figure is from (68).

1.3.4.2 Roles of sRNA in biofilm development in *P. aeruginosa*

To date, in *P. aeruginosa*, more than 200 sRNAs have been characterized, where they have been shown to function in carbon catabolite repression (CrcZ) (71), control of virulence gene expression (RsmY and RsmZ) (72-74), iron uptake and storage (PrrF1 and PrrF2) (75) and quorum sensing (QS) (PhrS) (76). Among those sRNAs, only a few have been reported to be involved in biofilm development and dispersal, and their potential role in NO induced dispersal has not been assessed so far. *SrbA*, which is encoded by a 239 bp gene *srbA*, located in an intergenic region upstream of the *aceA* gene in *P. aeruginosa* PA14, is important for biofilm formation since its expression level was 45 fold higher in biofilms compared to planktonic cells and the *srbA* mutant formed highly reduced biofilms relative to the WT (77). A sRNA PhrS, whose transcription depends on the O₂ responsive regulator ANR, was identified as an activator of the synthesis of QS regulator PqsR (76), which indicates that PhrS may be involved in O₂-depletion induced or NO induced biofilm dispersal. sRNAs RsmY and RsmZ, which can bind and reduce the availability of the protein RsmA (72, 74, 78, 79), play important roles in biofilm formation since RsmA represses *psl* and QS related genes that are important for biofilm formation (80, 81). A novel RsmY/RsmZ-type sRNA, RsmW, was upregulated in *P. aeruginosa* PAO1 biofilms compared to planktonic cells as well as being highly induced at high temperature (82). The expression levels of the sRNAs RsmY and RsmZ were reported to be lower in NO dispersed cells compared to planktonic cells, indicating a role of sRNAs in regulating biofilm formation in *P. aeruginosa* (1). However, in that study, the profile of sRNAs that were smaller than 100 nucleotides was missing due to

technical limitations of the sequencing methods used. Hence, the roles of most sRNAs in NO induced biofilm dispersal remain unclear.

Overall, it is hypothesized here that, in addition to modulating c-di-GMP, NO may indirectly impact on biofilm development via other pathways such as controlling iron uptake system and inducing sRNA regulation. To test those hypotheses, in the present study, a global, non-targeted approach investigating the change in both transcriptome as well as sRNA expression was undertaken. Profiles of mRNA and sRNAs that are bigger than 17 nt with and without NO treatment have been compared.

1.3.5 Application of NO as biofilm control strategy: Promising prospects and problems

NO is a very promising strategy for biofilm control in both clinical and industrial contexts. As mentioned above, biofilm-related infections are highly diverse since biofilms can form on a variety of living tissues as well as abiotic surfaces such as contact lenses (13). NO based anti-biofilm strategies offer a novel approach to improve standard antibiotic therapies through combined administration of NO and antibiotics. However, the implementation of NO for medical and industrial applications has been challenging due to its short half-life, which is only a few seconds in biological systems (83). To date, a variety of NO delivery methods have been studied and used to improve the effectiveness of NO. For example, a range of different NO donor compounds are already available. The effect of different NO compounds (e.g., SNP, S-nitrosoglutathione (GSNO) or the diazeniumdiolates (NONOates)) on biofilms formed by different bacteria species and mixed species biofilms has been well summarised in previous reviews (36, 84). Additionally, inhaled NO gas has

been approved by the US Food and Drug Administration (FDA) and the European Medicine Evaluation Agency and European Commission as a therapeutic agent (35). Furthermore, nanoparticles that release different levels of NO have shown effectiveness against biofilms (85). A more complete review of NO delivery methods, which is beyond the scope of this literature review, are described in (35).

It has also been shown that NO induced biofilm dispersal has industrially relevant applications. For example, the NO donor compound, MAHMA NONOate, was shown to disperse biofilms of isolates extracted from the membranes of reverse osmosis (RO) and membrane bioreactors (MBR) (86). The dispersal percentages ranged from 7 to 35% of isolates from RO membrane and from 4 to 40% for MBR isolates, suggesting a promising role of NO in removing biofilms from bioreactors. However, significant portions of biofilms still remained attached after exposure to NO and repeated treatments with NO do not improve biofilm dispersal (86). To better understand this phenomenon, in the current study, the response of biofilm cells to repeated exposures of NO in *P. aeruginosa* has been investigated. It was found that pretreatment of biofilms with very low concentrations of NO inhibited the subsequent dispersal response when exposed to higher concentrations of the signaling molecule.

1.4 The role of iron in *P. aeruginosa* biofilm development

In this thesis, the role of iron in *P. aeruginosa* biofilm development has been studied. Iron is an essential nutrient for almost all bacteria to grow, and bacteria have adapted several mechanisms to ensure sufficient uptake of iron from the

environment (87). Further, the homeostasis of intracellular iron in bacteria is strictly monitored because a high level of free iron can generate highly reactive and damaging oxidative free radicals via interacting with oxygen and oxygen-reduction products (87, 88).

1.4.1 Iron uptake and metabolism in *P. aeruginosa*

Gram-negative bacteria typically use two uptake systems to scavenge iron from the environment: the heme and pyoverdine uptake systems (89). In *P. aeruginosa*, two distinct heme uptake systems, Has and Phu, have been previously reported (90). In the Has system, cells secrete HasA_p, which is an extracellular hemophore protein, to take heme from hemoproteins and shuttle it to the HasR receptor (91). In the Phu system, hemoproteins bind the PhuR receptor and heme is extracted (89, 90). Under iron restricting conditions, many bacteria and fungi synthesize and secrete siderophores, which are ferric iron specific chelating agents, to sequester iron from the environment (92). *P. aeruginosa* produces two types of siderophores: the high-affinity pyoverdine and the lower-affinity pyocheline (93-97). As reviewed previously, in *P. aeruginosa*, the pyoverdine precursor ferribactin is made in the cytoplasm and exported to the periplasm to form mature pyoverdine via PvdO, PvdN, PvdM and PvdP (Figure 1.3) (89). The acylated ferribactin is probably deacylated by the PvdQ acylase when transported to the periplasm. Secreted pyoverdine binds Fe³⁺ in the extracellular space and the complex is taken up by the TonB-dependent receptor FpvA. The TonB protein, which spans the periplasm, facilitates siderophore transport through contacting with outer membrane receptors. Fe³⁺ is released from the pyoverdine-iron complex and is reduced to the Fe²⁺ form after being transported to the periplasm. Pyoverdine is then

exported to the extracellular space again via an efflux pump constituted by PvdT, PvdR and OpmQ.

Figure 1.3. Pyoverdine biosynthesis and uptake in *P. aeruginosa*. The figure is from (89). The non-fluorescent precursor of pyoverdine, ferribactin (white oval), is made in the cytoplasm and is transformed to a mature pyoverdine chromophore (green oval) in the periplasm. Secreted pyoverdine binds Fe^{3+} in the extracellular space to form a complex (green oval with a red dot) and the complex is taken up by the TonB-dependent receptor FpvA. After being back to the periplasm, Fe^{3+} is released from the complex and pyoverdine (green oval) is excreted to the extracellular space again.

The ferric uptake regulator (Fur) is a conserved protein in different Gram-negative bacteria that can directly control genes involved in iron uptake, or indirectly, via extracytoplasmic function sigma factors (ECF- σ) or via other regulators (e.g., the AraC-type regulator PchR). Fur works as a repressor of the

iron uptake genes when binds to its co-repressor Fe^{2+} and forms the active complex FeFur, which can bind to specific DNA sequences that located in the promoter region of iron acquisition genes and further represses downstream genes through blocking the access to DNA polymerase (98-100). ECF- σ factors, which are often responsive to environmental stimuli, are needed for the transcription of different genes. The genes encoding the ECF- σ factors are adjacent to a gene encoding a transmembrane anti- σ factor and a gene encoding a TonB-dependent receptor (101).

In *P. aeruginosa*, 10 ECF- σ have been found to be repressed by Fur: PA0149, PA0472 (*fiuI*), PA1300, PA1912 (*femI*), PA2050, PA2387 (*fpvI*), PA2426 (*pvdS*), PA2468 (*foxI*), PA3410 (*hasR*) and PA3899 (*fecA*) (102). *pvdS* codes for the ECF- σ factor required to activate pyoverdine synthesis and is involved in the transcriptional control of virulence factors endotoxin A and protease PrpL (103). FpvI is involved in the transcription of the TonB-dependent ferripyoverdine receptor gene *fpvA* (104). The regulator of pyochelin biosynthesis, *pchR*, is also repressed by Fur (105). Genes that are directly or indirectly controlled by Fur have been summarized by Cornelis *et al.* (101) (Figure 1.4). Additionally, Fur activates gene expression indirectly through control of a pair of sRNAs, PrrF1 and PrrF2, which are mRNA anti-sense, post-transcriptional regulators that induce upregulation of genes involved in iron storage, oxidative stress resistance and intermediary metabolism (75, 106).

1.4.2 The roles of iron and siderophores in biofilm development

As an important environmental signal in biofilm development, iron has been reported to control biofilm formation via modulating QS, effecting DNA release and enhancing production of PSL (33, 108, 109). The effect of iron replete and depleted conditions on *P. aeruginosa* biofilms and mechanisms are summarized in Table 1.1.

Although the involvement of iron in biofilm development has been studied for many years, the mechanisms of iron in inducing or affecting biofilm formation have not been clearly elucidated. QS and c-di-GMP are two of the main pathways that drive the transition between biofilms and planktonic cells in *P. aeruginosa* (21, 110). The expression of *lasIR* and *rhlIR*, which encode the major QS network in *P. aeruginosa* (111), are known to be enhanced by iron limiting conditions and repressed by iron supplementation (112-116). Furthermore, RhII was found to be involved in iron limitation induced twitching motility (117). 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), which is a QS signal, was found to function as an iron chelator and to induce the expression of genes involved in the regulation and synthesis of pyoverdine and pyocheline (118). Therefore, iron may effect biofilm formation via QS. However, further studies are needed to clarify the link between iron and QS in biofilm development.

Table 1.1. The effect of different iron conditions on biofilms.

Strains	Iron condition	Biofilm system	Results	Mechanisms	References
<i>P. aeruginosa</i> PAO1	Iron-limiting condition	Flow cell	Bacterial surface motility increases; Form flat unstructured biofilms	Induce rhamnolipid production and <i>rhlA</i> expression	(33)
<i>P. aeruginosa</i> PAO1 $\Delta pvdA$, $\Delta pvdS$ and $\Delta fpvA$	Without sufficient iron	Flow cell	Form thin and flat biofilms rather than mushroom-like structures	Pyoverdine mutants do not have sufficient intracellular iron for biofilm development	(97)
<i>P. aeruginosa</i> PA14 $\Delta pvdQ$	Iron-limiting condition	96 well plates	No biofilm formation	Unclear	(119)
<i>P. aeruginosa</i> PAO1 <i>muc</i> mutants	Iron-limiting condition ($\leq 5 \mu\text{M}$)	Colonies grown on agar plates	Enhance alginate production and mucoidy	Unclear	(120)
<i>P. aeruginosa</i> PAO1	8–50 μM FeCl_3 or 0.5–25 μM holo-transferrin	96 well tissue culture plates	Biofilm formation increases	Unclear	(34)
<i>P. aeruginosa</i> PAO1	5–100 μM FeCl_3	Microtitre trays	Biofilm formation and extracellular DNA decrease with increased iron concentration	Low iron (1 μM FeCl_3) induces the expression of the <i>pqs</i> operon and high iron (100 μM FeCl_3) represses the expression	(109)
<i>P. aeruginosa</i> PAO1	100 μM FeCl_3	Glass coverslips in glass chambers	Induce biofilm formation via elevating PSL production	Iron reduces rhamnolipid biosynthesis and inhibits the expression of AmrZ	(108)

Siderophores were found to be important for biofilm formation in *P. aeruginosa* (97). Siderophore production was shown to be regulated by the Gac/Rsm system, which plays a crucial role in the transition from planktonic cells to biofilms, through modulation of c-di-GMP levels rather than through Fur-mediated transcriptional control (78, 121). In the Gac/Rsm signaling network, GacA is activated by sensor kinases in response to environmental signals or cell-produced cues, and subsequently induce the transcription of sRNAs RsmY and RsmZ, which inhibit the protein RsmA by titration (72, 74, 78, 79). Several studies have shown that RsmA represses the translation of the biofilm EPS component PSL as well as inhibits the production of pyoverdine and pyochelin through modulating c-di-GMP levels (81, 121). The RsmA mediated inhibition of siderophore production was found to occur in the presence of different iron concentrations, from 0 to 50 μM FeCl_3 , as well as in the presence of 0 to 200 μM of the iron chelator 2,2'- Bipyridine (Bipy) (121). Thus, the regulatory effect of RsmA on pyoverdine via the Gac/Rsm system was suggested to be iron-independent. Another study showed that in the presence of high c-di-GMP, PEL and PSL exopolysaccharides positively regulate the expression of sRNAs RsmY and RsmZ and therefore chelate RsmA, which then induces the activity of SadC and promotes pyoverdine biosynthesis (122). c-di-GMP has been shown to be an intermediate between RsmA and pyoverdine (121), while another study suggests that c-di-GMP acts upstream of RsmA through PEL and PSL (122). This describes a possible feedforward loop between c-di-GMP and PSL. This link has been supported by another similar study showing that PSL promotes c-di-GMP production through stimulating SadC and SiaD and elevated c-di-GMP induces the production of PSL (123).

A recent study showed that in *P. aeruginosa*, high iron (50 and 100 μM FeCl_3) promoted PSL production and induced biofilm formation (108). PSL was also found to bind both ferrous and ferric iron and store iron to further induce PSL-dependent biofilm formation (108). However, it was suggested that c-di-GMP was not involved in iron-induced PSL production since the addition of iron had no impact on c-di-GMP levels. The authors further demonstrated that high iron promoted PSL production through repression of *rhlAB*, *rhlI* and *rhlR* transcription, thus reducing the synthesis of rhamnolipids as well as inhibiting *amrZ*, which encodes a transcriptional factor that inhibits the transcription of the *psl* operon (124-126).

1.4.3 Interplay between NO and iron and their roles in *P. aeruginosa* biofilm development

NO may directly or indirectly affect iron uptake. It has been previously shown that pyoverdine production is inhibited in NO treated cells in *P. aeruginosa* (26). Iron acquisition mechanisms can be inhibited by NO (in the micro molar range) through inactivation of Fur (127). As mentioned above, Fur acts as a transcriptional repressor of the iron uptake genes when it binds the co-repressor Fe^{2+} (98). NO was shown to interfere with the DNA-binding activity of Fur in *E. coli* through reacting with Fe^{2+} ions in the FeFur complex and forming an inactive and stable iron nitrosyl adduct (127). Further, in *E. coli*, the repressor activity of Fur on a Fur-regulated gene, *fhuF*, was shown to be inhibited by NO (127). Iron also affects NO through Fur. The NO detoxifying enzyme, Hmp, which can aerobically metabolize $\text{NO}^\cdot$ to NO_3^- by using O_2 and NADPH, was found to be regulated by Fur in *Salmonella typhimurium* (128). In *E. coli*, *hmp* expression was induced upon the addition of an iron chelator (129). In *P.*

aeruginosa, the Hmp-homologue flavohemoglobin is Fhp (47). Whether the gene encoding flavohemoglobin Fhp, *fhp*, is regulated by Fur in *P. aeruginosa* remains unclear.

As mentioned above, both iron and NO can serve as environmental signals for biofilm development through multiple pathways. To summarise, a schematic diagram that describes the link between, iron, NO, c-di-GMP, QS and their roles in the transition between biofilms and planktonic cells is shown in Figure 1.5.

Figure 1.5. Model depicting the link between the iron, NO, c-di-GMP, QS and their roles in the transition between biofilms and planktonic cells. The schematic is adapted from (121, 122). See text for details.

The interplay between iron and NO in biofilm development is still unclear. To explore the underlying mechanisms, the effect of iron on NO induced dispersal was investigated in this thesis and it was found that iron overrides NO induced biofilm dispersal through the promotion of rapid attachment of planktonic cells,

which is a PSL-dependent phenotype. Moreover, the effect of NO on biofilms formed in the presence of iron has been investigated. It is found that NO can disperse freshly attached cells that were induced to attach in response to iron. In *P. aeruginosa*, NO disperses biofilms via modulating c-di-GMP which has been reported to regulate PSL production at both transcriptional and translation level (23). Therefore, it is hypothesized that both iron and NO influence *P. aeruginosa* biofilms through modulating PSL. This study links two important environmental signals (iron and NO) with their roles in biofilm development and discusses new ways for improving the use of NO in biofilm control strategies.

1.4.4 The impact of iron on biofilm control

In natural environments, bacteria can encounter varying levels of available iron. For example in wastewater iron is present at an average of 9 μM (130), while in the CF lung, the iron concentration varies from 2 to 130 μM (131-135). Understanding the role of iron in biofilm development is of great importance for biofilm control in both industrial and clinical settings. Iron homeostasis, particularly in the context of biofilm formation, has become an attractive target for therapeutic interventions and iron-chelation therapy could be a promising anti-biofilm strategy. The application of a range of iron-chelating compounds for biofilm control has been studied previously. For example, the addition of 50 mM EDTA, a divalent cation chelator, was reported to reduce the number of *P. aeruginosa* biofilm-associated cells by more than 99% (136). Lactoferrin, which is a protein of the immune system with a high affinity for iron and is present in tears (1-4 mg ml^{-1}) (137), airway secretions (0.4-1.0 mg ml^{-1}) (138, 139) and breast milk (3-7 mg ml^{-1}) (140), was found to prevent biofilm

formation of *P. aeruginosa* by stimulating bacterial twitching motility (141). *P. aeruginosa* biofilms were reduced by 42% and 99% using the Food and Drug Authority (USA) approved iron chelators deferoxamine and deferasirox, respectively (142). It has been reported that the combination of NO, iron chelator and tobramycin efficiently reduced the survival of the dispersed cells (1). Therefore, simultaneous treatment of biofilms with NO, an iron chelator and biocides could be a powerful way to disperse and kill biofilms in an iron-rich environment. In the current study, the effect of iron chelator Bipy was tested and it was observed that NO treated biofilms were more sensitive to iron limitation since the iron chelator and NO had synergetic effects in dispersing biofilms.

1.5 Aims and Objectives

The aims of this project are:

- To determine the NO dispersal response regulon by comparing transcriptomic profiles of both mRNA and sRNA for biofilms and planktonic cells.
- To investigate the interplay between two important environmental signals, iron and NO, and their roles in biofilm development.
- To determine the mechanism that prevents some biofilm cells from dispersing upon exposure to NO, which can be used to develop strategies to improve the application of NO as a biofilm control strategy.

Chapter Two: mRNA and sRNA Mediated Regulation of Nitric Oxide Induced Dispersal in *Pseudomonas aeruginosa*

2.1 Introduction

It has been observed that while the exogenous addition of nitric oxide (NO) can disperse a significant proportion of biofilms, NO generally does not disperse all of the cells within the biofilm (27). This incomplete dispersal has been observed for NO as well as for other dispersal cues, including quorum sensing (QS) inhibitors and starvation induced dispersal (143, 144). Additionally, reducing the intracellular bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) level via inducing the expression of a plasmid based phosphodiesterase also failed to disperse all of the biofilm (1), which might be due to the genetic and physiological heterogeneity of the cells within the biofilm (11). The physiological heterogeneity could also lead to altered activity of regulatory systems that operate in parallel to the dispersal response. For example, as introduced in Chapter 1, both the Gac/Rsm and c-di-GMP signaling networks control the transition between biofilms and planktonic cells in *Pseudomonas aeruginosa*. In the Gac/Rsm signaling network, GacA is activated by sensor kinases in response to environmental signals or cell-produced cues, and subsequently induces the transcription of sRNAs RsmY and RsmZ, which inhibit the protein RsmA by titration (72, 74, 78, 79). In the presence of high c-di-GMP, PEL and PSL exopolysaccharides positively regulate the expression of sRNAs RsmY and RsmZ and therefore chelate RsmA (122). Given the complexity underlying the genetic regulation of dispersal, it is

of considerable interest to study the interacting pathways involved in biofilm development with the aim of improving biofilm dispersal via promoting one pathway and inhibiting the other one.

Previous transcriptomic analyses (1, 27) showed that pyoverdine production was inhibited in NO treated cells. This suggests a potential link between iron uptake (pyoverdine production) and biofilm dispersal, although there is currently no direct evidence supporting this hypothesis. Additionally, the expression levels of the sRNAs RsmY and RsmZ can bind and reduce the availability of the protein RsmA (72, 74, 78, 79). They can also represses *psl* and QS related genes, which are important for biofilm formation (80, 81). This suggests a role for these two sRNAs in regulating biofilm formation in *P. aeruginosa* (1). Although the sRNAs RsmY and RsmZ have been reported to regulate biofilm development (1), the role of most sRNAs in NO induced biofilm dispersal remains unclear and has not been specifically addressed (1). Thus, there is a need to study the roles of other sRNAs in mediating dispersal since they have important roles in regulation of a variety of processes including the metabolic reactions and stress responses. In general, it is hypothesized that, in addition to modulating c-di-GMP, NO may impact biofilm development via other pathways such as controlling iron uptake (pyoverdine production) system and inducing sRNA regulation. To test those hypotheses, a global, non-targeted approach investigating the change in both transcriptome as well as sRNA expression was undertaken. To avoid the potential confounding issues of the commonly used NO donor sodium nitroprusside (SNP), which has an iron centre and potential toxicity due to cyanide production, spermine NONO ate (SP-NO) was used here as the NO donor.

2.2 Materials and Methods

2.2.1 Bacterial growth conditions

P. aeruginosa PAO1 wild type (WT) (145) was used in this study. *P. aeruginosa* mutant strains containing a transposon Tn5-derived insertion element (Tc^R) in genes that may be involved in NO induced dispersal were obtained from the University of Washington *P. aeruginosa* mutant library (supported by grant # NIH P30 DK089507). All bacterial strains used in this study are listed in Table A.1. Bacterial strains were grown routinely in Luria-Bertani (LB)-Miller (BD Difco) broth overnight at 37°C and 200 rpm for 16 h to prepare cells for experiments.

2.2.2 Biofilm-dispersal assay

Overnight cultures of *P. aeruginosa* PAO1 wild type and transposon mutants were diluted 200 fold in M9 minimal medium containing 9 mM NaCl (Merck), 22 mM KH_2PO_4 (Merck), 48 mM Na_2HPO_4 (Merck), 19 mM NH_4Cl (Merck), 2 mM $MgSO_4$ (Merck), 100 μM $CaCl_2$ (Merck) and 0.4% D (+)-Glucose anhydrous ($C_6H_{12}O_6$) (VWR). M9 medium was freshly prepared for every experiment. One ml of the diluted culture was added to 24 well microtitre plates (NUNC, Thermo Fisher Scientific). Cells were incubated at 37°C with shaking at 180 rpm for 6 h to form biofilms. After incubation, 100 μM SP-NO (Spermine NONO ate, Cayman Chemical) was added into designated wells and incubated for 15 min. The NO donor SP-NO was dissolved in 10 mM NaOH and was freshly prepared for every experiment. For the untreated control, an equal amount of 10 mM NaOH was added.

2.2.3 Generation of untreated planktonic cells, biofilms, remaining biofilms after NO treatment and NO dispersed cells

Overnight cultures of *P. aeruginosa* PAO1 were diluted 200 fold and grown in M9 medium in 24 well microtitre plates. Cells were incubated at 37°C with shaking at 180 rpm for 6 h or 5.75 h. Untreated planktonic (supernatant) cells (P) and biofilms (B) were collected from plates that were incubated for 6 h. For plates that were incubated for 5.75 h, biofilms were washed with PBS and were treated with fresh M9 medium in the presence of 100 µM SP-NO for 15 min. Subsequently, NO treated and remaining biofilms (RB) and dispersed cells (D) were collected, respectively. Untreated biofilms and remaining biofilms were collected from the bottom surface of each well using sterile cell scrapers and suspended in RNA protect reagent (Qiagen). After collection, all samples were centrifuged at 5, 000 g for 10 min and suspended in 1 ml RNA protect reagent (Qiagen) in Eppendorf tubes. Tubes with suspension were vortexed for 15 s and incubated at room temperature for 5 min before centrifuged at 5, 000 g for 10 min. Subsequently, supernatants in each tube were discarded and cell pellets were used for RNA extraction. This experiment was performed in triplicate.

2.2.4 Total RNA preparation

Total RNA was extracted from all samples using a Direct-zol™ RNA MiniPrep kit (Zymo Research) as per the manufacturer's protocol. DNase treatment was conducted using the TURBO DNA-free Kit (Ambion) as per the manufacturer's protocol. RNA was concentrated and purified using the RNA Clean & Concentrator™-5 kit (Zymo Research) as per the manufacturer's protocol. The integrity and concentration of the total RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit 2.0 Fluorometer (Invitrogen).

2.2.5 RNA sequencing and data analysis

Separate libraries were constructed for mRNAs and sRNAs. Gene expression analysis was conducted via High-throughput mRNA sequencing. For mRNA, the data was analysed using the TopHat and Cufflinks software (146). The sequence reads were mapped onto the *P. aeruginosa* PAO1 reference genome (downloaded from NCBI [NC_002516.2]). For sRNA, the data was analysed using Cutadapt-1.8.1 for QC/adaptor removal. sRNA reads were aligned against the *P. aeruginosa* PAO1 reference genome (downloaded from NCBI [NC_002516.2]) using bowtie2 (version 2.2.6) and the read counts were calculated using HTSeq. The small RNA sequences were downloaded from the Bacterial Small Regulatory RNA Database (BSRD) through the following link: www.bac-srna.org/BSRD/index.jsp#. Putative target genes for the sRNAs were found through the BSRD link.

2.3 Results & Discussion

In this chapter, the results section and the discussion section have been combined into a single narrative as they are closely connected.

2.3.1 Overview of mRNA profiles in NO treated and untreated cells

Gene expression levels were compared between four types of cells: untreated planktonic cells (P), untreated biofilms (B), biofilm cells remaining after NO treatment (RB) and NO dispersed cells (D). Significant differences in gene expression (fragments per kilo base of transcript per million fragments mapped (FPKM)) were determined using the statistical model of Cuffdiff (146). Principal component analysis (PCA) for all of the samples showed that replicates of every type of cells were well clustered (Figure 2.1). Table 2.1

presents the upregulated and downregulated genes between five pairs of conditions: B-RB, B-D, RB-D, P-D, P-B.

Figure 2.1 Principal component analysis (PCA) of gene expression profiles of *Pseudomonas aeruginosa* untreated planktonic cells and biofilms, remaining biofilms after NO treatment and NO dispersed cells.

Table 2.1 Number of upregulated and downregulated genes between five pairs of conditions: B-RB, B-D, RB-D, P-D, and P-B (with a P-value cut-off of 0.05 and a fold-change cut-off of 4).

Samples	Number of total changed genes	Number of upregulated genes	Number of downregulated genes
B VS* RB	271	114	157
B VS D	122	97	25
RB VS D	132	95	37
P VS D	180	160	20
P VS B	41	31	10

*Compared to B, number of genes that were upregulated/downregulated in RB.

P: untreated planktonic cells; B: untreated biofilms; RB: biofilm cells remaining after NO treatment; D: NO dispersed cells.

The top 20 genes with the greatest fold change between each pair of conditions were selected and are listed in Appendix A (Tables A.2-A.6). Among the top 20 genes that showed differential expression, *hutU* expression increased by 107.6 fold in the remaining biofilms (RB) after NO treatment compared to untreated biofilms (B) (Table A.2). The expression level of *hutU* was 25.1 fold higher in dispersed cells (D) compared to untreated planktonic cells (P) (Table A.5). In addition, *hutU* expression increased by 12.3 fold in dispersed cells after NO treatment compared to untreated biofilms. *hutU* is involved in the conversion of L-histidine to N-formimino-L-glutamate (147). To investigate whether this histidine degradation pathway is linked to NO induced biofilm dispersal, a transposon mutant harbouring a mutation in *hutU* was tested for any change in NO induced dispersal. However, this mutant showed no difference in dispersal compared to the wild type (Figure 2.2).

Figure 2.2. HutU is not involved in NO induced dispersal. Biofilms of *P. aeruginosa* wild type (A) and the transposon mutant $\Delta hutU$ (B) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 100 μ M spermine NONOate (SP-NO) for 15 min before crystal violet (CV) staining to quantify the amount of biofilm. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (****, $P < 0.0001$).

Moreover, expression levels of *liuR*, *liuA* and *liuB* in NO treated biofilms were found to be 29, 44 and 15 fold higher, respectively, than the expression levels in untreated biofilms (Table A.2). *liuA* and *liuB* are regulated by *liuR* and they are all involved in the leucine/isovalerate catabolic pathways (148). The gene cluster of *bkdA1*, *bkdA2*, *bkdB* and *lpdV*, which belongs to a dehydrogenase operon that encodes a multi-enzyme complex branched-chain keto acid dehydrogenase involved in metabolism of valine, leucine and isoleucine, was also highly induced in NO treated cells. *bkdA1/bkdA2* were upregulated by 91 and 56 fold in NO treated biofilms and NO dispersed cells compared to untreated biofilms (Tables A.2 and A.3). *lpdV* was upregulated by 44 fold in NO treated biofilms compared to untreated biofilms (Table A.2). The expression levels of *bkdA1/bkdA2* were 48 fold higher in dispersed cells compared to untreated planktonic cells (Table A.5). *bkdA1*, *bkdA2* and *lpdV* were also shown to be slightly upregulated in NO or YhjH dispersed cells compared to planktonic cells in a previous study (1). However, expression of those genes was highly reduced in biofilms compared to planktonic cells and dispersed cells. As shown previously, the *bkdA1A2B-lpdV* and *liuABCD* genes are important for the dynamics of the biofilms as they are upregulated in early biofilm formation but downregulated in mature biofilms (149-151). Taken together, it is possible that the leucine catabolic pathways are linked to the biofilm development and therefore we hypothesized that the induction of the *bkdA1A2B-lpdV* and *liuABCD* gene cluster is linked to NO induced dispersal in the current biofilm system. However, no difference in biofilm dispersal was found for mutants of the above genes and the *P. aeruginosa* PAO1 wild type (Figure 2.3). Although the *bkdA1A2B-lpdV* may not be required for NO induced

dispersal, they may act downstream of NO as part of other pathways that are linked to biofilm dispersal, for example, under control of c-di-GMP. One possibility is that NO induced dispersal stimulates the cells to become metabolically active in preparation for dispersal and the subsequent colonisation of new sites.

Figure 2.3. The *bkdA1A2B-lpdV* cluster is not involved in NO induced dispersal. Biofilms of *P. aeruginosa* wild type (A) and transposon mutants including $\Delta liuA$ (B), $\Delta liuR$ (C), $\Delta bkdA1$ (D), $\Delta bkdA2$ (E), $\Delta bkdR$ (F) and $\Delta lpdV$ (G) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 100 μ M SP-NO for 15 min before analysed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Furthermore, among the top 20 genes that showed differential expression, expression levels of the NO detoxifying (aerobically metabolizes $\text{NO}^\cdot$ to NO^3 - by using O_2 and NADPH (152)) flavohemoprotein-encoding gene, *fhp*, were

105.3 fold higher in NO treated compared to untreated biofilms (Table A.2) and 162.9 fold higher in dispersed cells compared to untreated planktonic cells (Table A.5). The role of *fhp* in NO induced dispersal of *P. aeruginosa* biofilms and its expression under different conditions have been investigated in greater detail and are presented in Chapter 4.

2.3.2 The cells remaining within biofilms after dispersal and dispersed cells have different transcriptomic profiles

To investigate the reason why a portion of the biofilms always remained after NO induced dispersal, the gene expression of remaining biofilms after NO treatment and NO dispersed cells were compared. The top 20 upregulated and downregulated genes in the dispersed cells compared to remaining biofilms are listed in Table A.4. To determine if those genes were involved in the dispersal resistance of the remaining biofilms to NO treatment, transposon mutant strains of all of those genes were tested using the dispersal assay. Mutants of target genes are expected to be more sensitive to a low dose of NO (10 μ M SP-NO) or can be entirely dispersed by the high dose of NO (100 μ M SP-NO). However, all mutants showed the same phenotype as the wild type when treated with 10 or 100 μ M SP-NO (Figures 2.4-2.10), suggesting that they may not contribute to the dispersal resistance of the remaining biofilms. Several reasons may account for the negative results. First, investigating only the top 20 genes that showed significantly different expression levels between remaining biofilms and dispersed cells may miss out on key genes that are directly involved in the NO dispersal pathway. Further pathway analysis, for example, the Gene Set Enrichment Analysis (GSEA), could be performed to identify important genes or a group of genes. Moreover, it is possible that the NO response is a multi-

gene phenotype, requiring more than one protein to be deleted to have a measurable phenotype. In the future, it would be interesting to start from studying the protein-protein interaction of suspected proteins by using methods such as the pull-down assay (153), which first immobilizes the suspected protein on an affinity ligand specific to the tag and creates an affinity support to capture and purify other proteins from cell lysates that interact with the suspected protein. Next step is to study the response of those interacted proteins to NO as the interacted complex may contain NO-sensing proteins or NO-scavenging proteins.

Figure 2.4. Genes that do not contribute to the resistance of remaining biofilms after NO treatment-1. Biofilms of *P. aeruginosa* wild type (A) and transposon mutants including ΔPA4155 (B), ΔfemR (C), ΔfemI (D), ΔPA1923 (E) and ΔPA1925 (F) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 10 or 100 μM SP-NO for 15 min before analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (*, P < 0.1; ***, P < 0.001; ****, P < 0.0001).

Figure 2.5. Genes that do not contribute to the resistance of remaining biofilms after NO treatment-2. Biofilms of *P. aeruginosa* transposon mutants including ΔPA0433 (A), ΔPA4837 (B), ΔPA3600 (C), ΔpvdQ (D), ΔPA5059 (E) and ΔpyrQ (F) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 10 or 100 μM SP-NO for 15 min before analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (***, $P < 0.001$; ****, $P < 0.0001$).

Figure 2.6. Genes that do not contribute to the resistance of remaining biofilms after NO treatment-3. Biofilms of *P. aeruginosa* transposon mutants including ΔPA2366 (A), ΔPA4834 (B), ΔPA4835 (C), ΔPA4836 (D), ΔPA0434 (E) and ΔPA2034 (F) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 10 or 100 μM SP-NO for 15 min before analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (***, P < 0.001; ****, P < 0.0001).

Figure 2.7. Genes that do not contribute to the resistance of remaining biofilms after NO treatment-4. Biofilms of *P. aeruginosa* transposon mutants including $\Delta cyoC$ and $\Delta tadD$ were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 10 or 100 μ M SP-NO for 15 min before analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (*, P < 0.1; ****, P < 0.0001).

Figure 2.8. Genes that do not contribute to the resistance of remaining biofilms after NO treatment-5. Biofilms of *P. aeruginosa* wild type (A) and transposon mutants including ΔPA1539 (B), ΔPA3009 (C), ΔhutU (D), ΔlpdV (E) and ΔPA4683 (F) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 100 μM SP-NO for 15 min before analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (**, P < 0.01; ****, P < 0.0001).

Figure 2.9. Genes that do not contribute to the resistance of remaining biofilms after NO treatment-6. Biofilms of *P. aeruginosa* transposon mutants including Δ PA2555 (A), *liuA* (B), Δ PA2224 (C), Δ PA3052 (D), Δ PA0840 (E), Δ map (F) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 100 μ M SP-NO for 15 min before analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (***, $P < 0.001$; ****, $P < 0.0001$).

Figure 2.10. Genes that do not contribute to the resistance of remaining biofilms after NO treatment-7. Biofilms of *P. aeruginosa* transposon mutants including $\Delta nuoA$ (A) and $\Delta PA1964$ (B) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 100 μ M SP-NO for 15 min before analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (****, P < 0.0001).

2.3.3 Overview of sRNA profiles in NO treated and untreated cells

The expression levels of sRNAs were compared between four types of cells: untreated planktonic (P), untreated biofilms (B), biofilm cells remaining after NO treatment (RB) and NO dispersed cells (D). Significant differences in sRNA expression levels were identified using HTSeq, where differences in sRNA expression were compared for five pairs of conditions: B-RB, B-D, RB-D, P-D and P-B. Principal component analysis (PCA) showed that replicates of each treatment type were clustered together (Figure 2.11).

Figure 2.11 PCA of sRNAs of *P. aeruginosa* untreated planktonic cells and biofilms, remaining biofilms after NO treatment and NO dispersed cells.

In total, 117 sRNAs could be aligned according to the BSRD database. Among those sRNAs, 64 sRNAs showed significantly different expression between the five pairs of conditions: B-RB, B-D, RB-D, P-D and P-B. A more detailed analysis, comparing the significantly upregulated and downregulated (>3 fold) sRNAs indicated that there are some overlaps of significantly changed sRNAs between the remaining biofilms after NO treatment and NO dispersed cells (compared to untreated cells) (Table 2.2). For example, SPA0077 was upregulated in NO treated biofilms compared to untreated biofilms and also showed a higher expression level in dispersed cells.

Table 2.2 Upregulated and downregulated sRNAs between five pairs of conditions: B-RB, B-D, RB-D, P-D, and P-B (with a P-value cut-off of 0.05 and a fold-change cut-off of 3).

	Upregulated sRNAs	Downregulated sRNAs
B VS* RB	SPA0077, SPA0165, SPA0114	P11
B VS D	SPA0077, pant66, SPA0100, SPA0165 and SPA0086	SPA0056, SPA0092, P31, SPA0071,
RB VS D	--	P15, SPA0071, SPA0114
P VS D	SPA0077, SPA0150, pant66, SPA0100, SPA0086, SPA0165, SPA0163, SPA0131	P15, P11, PhrX, SPA0027
P VS B	sRNA622	SPA0167

*Compared to B, sRNAs that are upregulated or downregulated in RB

P: untreated planktonic cells; B: untreated biofilms; RB: biofilm cells remaining after NO treatment; D: NO dispersed cells

2.3.4 Expression profiles of known sRNAs that are involved in biofilm development

In *P. aeruginosa*, RsmY and RsmZ regulate the translational repressor protein RsmA. RsmA is known to stimulate expression of genes required during acute infection (type III secretion, type IV pilus synthesis and flagellar motility) and to negatively regulate chronic infection related genes (secondary metabolites, siderophore, type VI secretion, extracellular enzymes) (154). It has been reported that the expression level of the small non-coding RNA RsmY was lower in biofilm-dispersed cells compared with planktonic cells (1). However, RNA sequencing data showed that *rsmY* and *rsmZ* were significantly upregulated in dispersed cells compared to planktonic cells before dispersal. *rsmY* was upregulated in both dispersed cells and in the treated biofilms

compared to untreated biofilms. Therefore, in the current study, *rsmY* and *rsmZ* may not be involved in SP-NO induced dispersal.

CrcZ has been shown to antagonise the function of Crc (catabolite repression control protein) in catabolite repression. The RNA binding protein Crc was reported to be central in the regulation of virulence and virulence-related processes (155). A *P. aeruginosa* *crc* mutant was impaired in twitching motility, type III secretion and biofilm formation (154). It was shown that *crcZ* was upregulated and *crc* was downregulated in dispersed cells compared to untreated planktonic cells. It was hypothesized that NO may disperse biofilms through promoting expression of *crcZ* and therefore affects the function of Crc. PrrF1 and PrrF2 are encoded in tandem and are preceded by a Fur binding site. These two sRNAs are produced during iron limitation (154). It was reported that Fur activates gene expression indirectly through control of PrrF1 and PrrF2. In addition, PrrF1 and PrrF2 were reported to positively regulate production of the PQS signaling molecule (156). Expression levels of PrrF2 were significantly lower in dispersed cells and NO treated biofilms compared to untreated biofilms. PrrF1 was downregulated in NO dispersed cells compared to planktonic cells. It has been reported that the $\Delta prrF1$ -F2 mutant is defective for both iron and homeostasis (156). Thus, NO may impact on iron homeostasis via modulating sRNAs PrrF1/PrrF2 and further induce biofilm dispersal.

2.3.5 Potential sRNA candidates that may be involved in NO induced biofilm dispersal were selected in the current study

Except for the known sRNAs that are involved in biofilm development, i.e. RsmYZ, additional potential sRNA candidates that may be involved in NO induced dispersal were selected based on two criteria and are discussed below.

1) The sRNA was significantly upregulated or downregulated in free-living cells including dispersed cells and planktonic cells compared to biofilms. 2) The sRNA was significantly upregulated or downregulated in NO treated cells compared to untreated cells.

P15 showed distinct expression levels in all four types of cells. Compared to untreated biofilms, P15 was upregulated in NO treated biofilms and was downregulated in dispersed cells. P15 is a *trans*-encoded antisense RNA and is located between PA2942 and PA2943 (BSRD). Computationally predicted target genes of P15 include *aer*, *pepP*, *pcm*, *copR*, *fliJ* and *aroC* (BSRD). According to the mRNA-Seq data, *pepP* exhibited lower expression level in planktonic cells and dispersed cells compared to biofilms. In *P. aeruginosa* PA14, the $\Delta pepP$ mutant produced elevated levels of pyocyanin and had somewhat reduced swimming ability (157). *pcm* was downregulated in dispersed cells compared to planktonic cells. The open reading frame *pcm* gene was found in the upstream region of *rpoS*, and in the same order as in *E. coli* (158). RpoS is a positive transcriptional regulator of *psl* gene expression in *P. aeruginosa* (81). Therefore, NO may influence PSL production via controlling P15 and *pcm*. *aroC* showed the lowest expression level in dispersed cells compared to other types of cells. Deletion of *aroC* caused deficiency in biofilm formation of *P. aeruginosa* PA14 (159). Overall, P15 could be an important sRNA that regulates NO induced dispersal through interacts with its predicted target genes including *pepP*, *pcm* and *aroC*. Further experiments (as mentioned below) are needed to validate the role of P15 and other genes.

The sRNAs SPA0071, SPA0114 and SPA0092 showed lower expression levels in dispersed cells compared to biofilms after NO treatment. Expression levels

of those three sRNAs were also lower in planktonic cells compared to untreated biofilms. Generally, the expression levels of those sRNAs were lower in planktonic cells and NO dispersed cells, suggesting that they are inhibited by NO treatment and are linked to biofilm dispersal. SPA0071 is a *trans*-encoded antisense RNA and is located between PA0805 and PA0806 (BSRD) and no target genes have yet been identified for this sRNA. SPA0114 is a *cis*-encoded antisense RNA, co-located with *nuoA* (BSRD). The gene *nuoA* (PA2637) encodes chain A of the proton-pumping NADH dehydrogenase I. mRNA data showed that the pattern of expression of *nuoA* was similar to SPA0114 in different types of cells. In *Pseudomonas fluorescens*, *nuo* mutants are deficient in plant root colonization (160), suggesting that NADH dehydrogenase complex I may be involved in aerobic motility and biofilm formation (161). Thus, SPA0114 may regulate biofilm formation through control of SPA0114 and *nuoA*. However, the transposon mutant strain *nuoA* showed a similar dispersal response as the wild type (Figure 2.10A), indicating this gene may be not involved in NO induced dispersal. SPA0092 is a *trans*-encoded antisense RNA and is located with *crcZ* (BSRD) and no target genes controlled by this sRNA have been identified to date. SPA0167 is a *cis*-encoded antisense RNA and it is located between *pcpS* and PA1166. It has an overlap with both *pcpS* and PA1166. *pcpS*, encoding the multifunctional phosphopantetheinyl transferase of *P. aeruginosa*, plays an essential role in both fatty acid and siderophore metabolism. The *pcpS* mutant is impaired in siderophore synthesis (162). Given that NO treatment resulted in reduced pyoverdine production, NO may control expression of the *pvd* genes through altered expression of SPA0167 and *pcpS*.

Pant66, SPA0100, SPA0077 showed at least 5 fold higher expression in dispersed cells compared to planktonic cells, while their expression levels were similar in biofilm and planktonic cells. Thus, they are probably responsive to NO but may not be involved in regulating biofilm development. P11 was downregulated (4.2 fold) in NO treated biofilms compared to untreated biofilms. Additionally, P11 showed a 4 fold lower expression level in dispersed cells compared to planktonic cells. P11, also annotated as NalA (nitrogen assimilation leader A), represents the leader RNA of the *nirBD-PA1779-cobA* operon. NalA transcription is σ^{54} and NtrC dependent. A *nalA* deletion strain failed to grow on nitrate, although it had no influence on *P. aeruginosa* PAO1 motility and biofilm formation (163). Thus, P11 might be responsive to NO rather than being involved in NO induced biofilm dispersal.

Overall, sRNA candidates that may be involved in NO induced dispersal have been selected based on the two criteria as mentioned above and are listed in Table 2.3. Their roles in biofilm development and NO induced dispersal will be further studied in experiments as following. Firstly, expression levels of target sRNAs will be validated by real-time quantitative reverse transcription PCR (qRT-PCR) experiment. Deletion mutants will be made so that their biofilm formation phenotypes as well as their response to NO can be determined. After that, target mRNAs of those validated sRNAs will be predicated through computational models including TargetRNA, sRNAtarget, IntaRNA, RNApredator and sTarPicker. Further, the expression levels of predicated target mRNAs of validated sRNA will be detected in deletion or overexpressed mutants of sRNA using qRT-PCR.

Table 2.3 sRNA genes that were altered in expression upon NO addition.

Name	Types of sRNAs	Predicted target genes	Flanking genes
P15	<i>Trans</i> -encoded antisense RNA	<i>aer</i> , <i>pepP</i> , <i>pcm</i> , <i>copR</i> , <i>fliJ</i> , <i>aroC</i>	PA2942/PA2943
SPA0071	<i>Trans</i> -encoded antisense RNA	Unknown	PA0805/PA0806
SPA0114	<i>Cis</i> -encoded antisense RNA	<i>nuoA</i>	<i>nuoA</i>
SPA0092	<i>Trans</i> -encoded antisense RNA	Unknown	<i>crcZ</i>
SPA0167	<i>Cis</i> -encoded antisense RNA	PA1166/ <i>pcpS</i>	PA1166/ <i>pcpS</i>
SPA0086	<i>Trans</i> -encoded antisense RNA	Unknown	PA3919/PA3920
SPA0131	<i>Trans</i> -encoded antisense RNA	Unknown	<i>hasAp/hasD</i>
SPA0165	<i>Cis</i> -encoded antisense RNA	PA1735	PA1735
SPA0163	<i>Cis</i> -encoded antisense RNA	PA2500	PA2500
P31	<i>Trans</i> -encoded antisense RNA	<i>ureE</i> , <i>hcnB</i> , <i>coaC</i> , <i>mucA</i> , <i>pcrD</i> , <i>rpsB</i>	<i>carA</i> /P32
PhrX	<i>Trans</i> -encoded antisense RNA	Unknown	PA5183/PA5184
P11	<i>Trans</i> -encoded antisense RNA	<i>pilF</i> , <i>popB</i> , <i>liuC</i> , <i>fhp</i> , <i>cbpD</i> , <i>dxr</i> , <i>murD</i> , <i>rho</i> , <i>wbpY</i>	<i>nirB</i> /PA1782
SPA0056	<i>Cis</i> -encoded antisense RNA	PA0708	PA0708
SPA0027	<i>Trans</i> -encoded antisense RNA	Unknown	<i>toxR</i> /PA0708

In summary, this study has successfully created transcriptomic profiles of both mRNA and small, non-coding RNA with and without NO treatment in *P. aeruginosa*. Transcriptomic profiles of the cells remaining within biofilms and dispersed cells were compared to develop hypotheses that why NO treatment does not disperse all biofilm cells. Additionally, the sRNA sequencing data highlighted several sRNA candidates that may be involved in NO induced dispersal. Further experiments including making deletion mutants of sRNA genes will be conducted to study the roles of sRNA candidates.

Chapter Three: The Roles of Iron and Nitric Oxide in Biofilm Development

3.1 Introduction

Biological life cycle transitions are often regulated by the interplay between genetic elements and chemical or environmental cues (164). Understanding these interactions may allow for a better control of developmental processes. In bacterial biofilms, 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 (165). The opportunistic pathogen and model 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 (166). In *P. aeruginosa*, alginate, PEL and PSL are partly controlled by bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), an intracellular secondary messenger conserved across bacterial species (21, 22). In most bacteria, c-di-GMP levels are controlled by multiple enzymes some of which are associated with sensory domains capable of responding to extracellular stimuli including environmental cues as well as cell to cell signals (21). While high levels of c-di-GMP, via interaction with transcript regulators and direct effectors, usually promote attachment, low intracellular levels

downregulate attachment, trigger dispersal and induce the expression of motility genes.

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) (26, 27), oxygen (O₂) depletion (28, 29), changes in temperature (30), changes in iron level and nutrient availability (31-34). Among these cues, NO has attracted particular interest as its role in biofilm dispersal 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 (84). NO is a hydrophobic molecule and a highly reactive free radical (36), and is active at low, non-toxic concentrations (nanomolar range) to induce dispersal, while in contrast higher concentrations may cause nitrosative damage to bacterial cells. In *P. aeruginosa* NO is known to disperse biofilms through stimulation of phosphodiesterase activity, decrease in intracellular c-di-GMP concentrations and the response requires the periplasmic protease LapG (167). 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 (168). While the exogenous addition of NO can disperse a significant portion of biofilms, the addition of NO generally does not disperse all of the biofilms (27). We have recently shown that the non-dispersing cells become insensitive to NO as a consequence of producing a flavohemoprotein capable of scavenging free NO signals (169).

One of the most important reactions in which NO participates in biological systems are those with iron, where NO can bind to heme sensors, affect cytochromes or iron-sulfur clusters (170). Interestingly, iron has been shown to impact biofilm developmental processes, but the 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, which may be especially important in conditions of high cellular density such as biofilms (87). Mature biofilms have been found to exhibit gene expression profiles consistent with iron limitation, and previous studies reported that iron availability can control biofilm formation through several mechanisms, including modulating quorum sensing (QS) cell-cell signaling, stimulating DNA release, or enhancing the production of PSL polysaccharides (33, 108, 109). Generally, under iron-limiting conditions *P. aeruginosa* does not form biofilms or only forms flat, unstructured biofilms (33, 119). In contrast, under iron replete conditions, biofilm formation is increased (34, 108). Pyoverdine, the high-affinity siderophore that is produced by *P. aeruginosa* under iron-limiting conditions to acquire iron from the environment (92, 94, 95), is linked to biofilm formation and c-di-GMP. *P. aeruginosa* cells, which c-di-GMP levels were reduced by genetic means, showed reduced levels of pyoverdine compared to control cells (1, 121, 171). The mechanisms regulating these effects remain to be fully elucidated and, so far, no c-di-GMP-dependent receptor involved in *pvd* transcription has been identified yet. Previous studies had also demonstrated that *P. aeruginosa* $\Delta pvdA$, $\Delta pvdS$ and $\Delta fpvA$ mutant strains, defective in genes that are important for pyoverdine synthesis, signaling and uptake (97, 172),

form thin biofilms and for the $\Delta pvdA$ mutant, the biofilm mushroom-like structure could be restored when pyoverdine was exogenously added (97). These observations further support the involvement of pyoverdine in biofilm development. Iron may also affect biofilm formation through the QS signaling pathway. RhlI, one of the signal synthases in the QS regulon of *P. aeruginosa*, was found to be involved in iron limitation-induced twitching motility (117). It was mentioned that RhlI⁺ strain forms biofilms poorly under iron limiting condition, while the structured, mushroom-like biofilm formation was largely restored in the *rhlI* mutant. Besides, a recent study showed that in *P. aeruginosa*, high iron (50 and 100 μ M FeCl₃) promoted PSL production and induced biofilm formation (28). PSL was also found to bind both ferrous and ferric iron and store iron to further induce PSL-dependent biofilm formation (108). Further, they demonstrated that high iron promoted PSL production through 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 (124-126).

In this study, gene expression profiles of *P. aeruginosa* biofilms and planktonic cells in the presence and absence of NO were compared through transcriptomic analysis. NO dispersed cells had reduced expression of pyoverdine and pyochelin synthesis genes as well as iron acquisition related genes. This suggests that the regulatory pathways for iron acquisition and NO mediated dispersal may be related. NO signals may disperse biofilms through controlling iron or iron uptake system, or alternatively, downregulation of iron acquisition may be a consequence of biofilm dispersal. To explore the interplay between

iron and NO and their effect on biofilm development, iron was added into NO treated cells and the effect of iron on NO induced dispersal has been investigated. It was found that iron could override NO induced biofilm dispersal through promoting the rapid attachment of planktonic cells and NO dispersed cells, which is a PSL-dependent phenotype. Moreover, NO was shown to disperse freshly formed biofilms that were induced by iron. Hence, the current results suggested that iron and NO both control biofilm development via parallel signaling pathways and both do so either directly or indirectly through modulating the biofilm matrix component PSL. Based on those findings, it was hypothesized that NO treated biofilms were more sensitive to iron limitation and it was found that the addition of an iron chelator in the presence of NO showed a synergetic effect on dispersing biofilms.

3.2 Materials and Methods

3.2.1 Bacteria and growth conditions

P. aeruginosa PAO1 wild type (WT) (145) was used in this study. *P. aeruginosa* mutant strains, including Δpel (isogenic *pelF* deletion mutant) (173), Δpsl (isogenic *pslA* deletion mutant) (173) and Δalg (isogenic *alg8* deletion mutant) (173) were also used. Bacteria were routinely grown in Luria-Bertani (LB)-Miller broth (BD Difco) overnight at 37°C with shaking at 200 rpm for 16 h to prepare cells for experiments.

3.2.2 Biofilm dispersal assays

Biofilms were grown as previously described (174) with some modifications. Briefly, overnight cultures of *P. aeruginosa* PAO1 wild type and mutants were diluted 200 fold to an OD₆₀₀ of 0.003 in M9 minimal medium (containing 9

mM NaCl, 22 mM KH₂PO₄, 48 mM Na₂HPO₄, 19 mM NH₄Cl, 2 mM MgSO₄, 100 µM CaCl₂ and 0.4% D-glucose, pH 7.0), which was freshly prepared for every experiment. One ml 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 at 37°C. After 6 h, the NO donor SP-NO (Spermine NONO ate, Cayman Chemical) was added into 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, biofilm biomass was analyzed by crystal violet (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 KH₂PO₄ and 10 mM Na₂HPO₄, 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 MilliQ water. Stained biofilms were washed twice with 1 ml PBS and the remaining CV was dissolved in 1 ml of absolute ethanol. Biofilm biomass was quantified by measurement of the absorbance at 550 nm (OD₅₅₀) in an Infinite Pro2000 microplate reader (Tecan). All biofilm assays were performed in independent, triplicate experiments.

3.2.3 Pyoverdine quantification

Production of pyoverdine in supernatants of *P. aeruginosa* overnight cultures was quantified by measuring fluorescence (excitation wavelength, 400 nm; emission wavelength, 450 nm; Infinite Pro2000 microplate reader, Tecan) and the pyoverdine production level was normalized by OD₆₀₀.

3.2.4 Planktonic cells rapid attachment assays

Biofilms were grown as described in biofilm dispersal assays. After 6 h, FeCl_3 , FeSO_4 , ZnCl_2 , MnCl_2 , CuSO_4 , H_2O_2 or the NO scavenging compound [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] (PTIO) was added into the cultures that had received or not NO treatment. The final concentration of metal salts or H_2O_2 varied from 100 nM to 1 mM and the concentration of PTIO was 200 μM . The plates were incubated for a further period from 5 min to 60 min. After the final incubation, biofilm biomass was quantified using CV staining as described above.

3.2.5 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. 100 μM FeSO_4 , 100 μM FeCl_3 or 200 μM PTIO was added 15 min after addition of NO.

3.2.6 Biofilm, PSL staining and 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 FeCl_3 or 100 μM NO donor for 30 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. 1.5 μL 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. PSL matrix was stained by 100 $\mu\text{g/mL}$ tetramethyl

rhodamine isothiocyanate (TRITC)-labelled hippeastrum hybrid lectin (amaryllis) (HHA) (EY Labs, Inc.), as previously described (20). TRITC-HHA was added 2 h prior to NO or iron treatment. 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).

3.2.7 Statistical analysis

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

3.3 Results

3.3.1 NO inhibits expression of iron acquisition genes and pyoverdine production

To elucidate the molecular pathway of NO induced dispersal and to identify putative NO binding receptors, this study has compared transcriptomic profiles of *P. aeruginosa* untreated planktonic cells and biofilms as well as the cells remain within biofilms after NO treatment and NO dispersed cells as shown in Chapter 2. Transcriptomics analyses showed that the expression levels of most iron acquisition related genes in NO treated biofilms and dispersed cells were lower compared to untreated cells (Table 3.1 and 3.2). Several extracytoplasmic function sigma factors (ECF- σ) controlled by the ferric uptake regulator (Fur) (101) including *pvdS* and *femI* were downregulated in NO treated cells (biofilms and dispersed cells) compared to untreated cells. Genes for pyoverdine synthesis appeared to be downregulated, such as *pvdA* (97), which

was 7 fold lower in NO treated biofilms compared to untreated biofilms and 5 fold lower in dispersed cells compared to planktonic cells. *tonB1*, an essential component in iron-siderophore uptake system (89) was also decreased by 11 and 4 fold in NO treated biofilms and dispersed cells, respectively, compared to untreated biofilms. Iron receptors, including *fpvA*, *optI* and *hasR* exhibited lower expression levels in NO treated biofilms and dispersed cells compared to untreated biofilms. Among these genes, the expression level of *fpvA* was 7 fold lower in NO treated biofilms compared to untreated biofilms. Moreover, the expression of *pvdQ* was nine times lower compared to untreated biofilms and *fptA*, *pchA-D*, *pchR* (105) which are involved in pyochelin biosynthesis were also downregulated in NO treated biofilms and dispersed cells compared to untreated biofilms. In addition, the expression levels of phenazine biosynthesis genes, which encode redox-active pigments that are involved in QS, virulence and iron acquisition (175), including *phzA1*, *phzC1*, *phzC2*, *phzD1*, *phzD2*, *phzE1* and *phzE2* were decreased at least 7 fold in NO treated biofilms. Therefore, the data suggests that the pyoverdine and pyochelin synthesis genes, as well as iron acquisition related genes, were generally reduced after NO treatment.

In contrast, *bfrB*, coding for a bacterioferritin, which is a main iron-storage protein in *P. aeruginosa*, was highly upregulated (72 fold) in NO treated and remaining biofilms compared to untreated biofilms (Table 3.1 and 3.2). Similar results were reported in response to SNP (176). A recent study revealed that iron accumulation by BfrB induces acute iron deprivation in the cytosol as well as the de-repression of iron acquisition genes. However, in our study, it was found that NO induced *bfrB* expression while repressing iron acquisition genes.

The contradictory findings relative to the previous study might be because that while *bfrB* is highly induced after NO treatment, it is possible that at the time point sampled, 15 min, most intracellular iron has not yet been bound by BfrB. Therefore, it is possible that most iron acquisition genes are still downregulated due to NO treatment and have not been de-repressed by BfrB. In our study, it was found that the *bfrB* mutant was dispersed to the same extent as the *P. aeruginosa* PAO1 wild type (Figure A.1), suggesting that BfrB does not contribute to biofilm dispersal. However, further work would be needed to better understand the exact mechanism for the differences between the work presented here and the previously published data.

Overall, it is possible that exogenous NO transmits a signal to *P. aeruginosa* cells that they have access to plenty of iron. This may explain why most iron acquisition genes were downregulated while the storage protein BfrB was induced in response to NO. It was also hypothesized that NO may impact on those genes through effect on the ferric uptake regulator Fur, which works as a repressor of those iron uptake genes. However, *fur* was shown to be downregulated (1.7 fold) in NO dispersed cells compared to untreated biofilms and there was no significant difference of expression level of *fur* between NO treated and untreated biofilms. In the future, it would be interesting to compare the expression of *fur* between untreated cells and NO treated cells again using real-time quantitative reverse transcription PCR (qRT-PCR).

Table 3.1 Genes that were changed in NO treated remaining biofilms compared to untreated biofilms (with a P-value cut-off of 0.05).

Gene	Fold change	COG
<i>bfrB</i>	71.8	Inorganic ion transport and metabolism
<i>femI</i>	-25.2	Transcription
<i>fagA</i>	-13.8	Not available
<i>fumC1</i>	-13.8	Energy production and conversion
<i>tonB1</i>	-11.4	Cell envelope biogenesis, outer membrane
<i>phuS</i>	-11.1	Inorganic ion transport and metabolism
PA4156	-9.5	Inorganic ion transport and metabolism
<i>pvdQ</i>	-9.1	General function prediction only
<i>phzC1</i>	-9.0	Amino acid transport and metabolism
<i>phzD1</i>	-9.0	Secondary metabolites biosynthesis, transport, and catabolism
<i>phzE1</i>	-9.0	Amino acid transport and metabolism / Coenzyme metabolism
<i>phzA1</i>	-8.0	Not available
<i>phzC2</i>	-7.6	Amino acid transport and metabolism
<i>phzD2</i>	-7.6	Secondary metabolites biosynthesis, transport, and catabolism
<i>phzE2</i>	-7.6	Amino acid transport and metabolism / Coenzyme metabolism
<i>fpvA</i>	-7.6	Inorganic ion transport and metabolism
<i>pvdA</i>	-7.3	Secondary metabolites biosynthesis, transport, and catabolism
<i>phuV</i>	-7.2	Inorganic ion transport and metabolism
<i>hasR</i>	-6.9	Inorganic ion transport and metabolism
PA1302	-6.8	Inorganic ion transport and metabolism
<i>foxR</i>	-6.5	Inorganic ion transport and metabolism / Signal transduction mechanisms
<i>pvdN</i>	-5.8	Posttranslational modification, protein turnover, chaperones
<i>phzG1</i>	-5.6	Coenzyme metabolism
<i>phzB2</i>	-5.5	Not available
<i>pvdO</i>	-5.1	Function unknown
<i>phzG2</i>	-5.1	Coenzyme metabolism
<i>pchA</i>	-5.1	Coenzyme metabolism / Secondary metabolites biosynthesis, transport, and catabolism
<i>pchB</i>	-5.1	Amino acid transport and metabolism
<i>pchC</i>	-5.1	Secondary metabolites biosynthesis, transport, and catabolism
<i>pchD</i>	-5.1	Secondary metabolites biosynthesis, transport, and catabolism
<i>fpvB</i>	-5.0	Inorganic ion transport and metabolism
<i>pchR</i>	-4.8	Transcription

Table 3.1 Genes that were changed in NO treated remaining biofilms compared to untreated biofilms (with a P-value cut-off of 0.05)(Cont'd).

Gene	Fold change	COG
<i>optI</i>	-4.5	Inorganic ion transport and metabolism
PA2407	-4.5	Inorganic ion transport and metabolism
PA2408	-4.5	Inorganic ion transport and metabolism
PA2409	-4.5	Inorganic ion transport and metabolism
PA2410	-4.5	Inorganic ion transport and metabolism
PA3410	-4.5	Transcription
<i>sodM</i>	-4.2	Inorganic ion transport and metabolism

Table 3.2 Genes that were changed in NO dispersed cells compared to untreated planktonic cells (with a P-value cut-off of 0.05).

Gene	Fold change	COG
<i>bfrB</i>	20.4	Inorganic ion transport and metabolism
<i>pvdA</i>	-5.6	Response to stimulus
<i>pvdS</i>	-5.3	Secondary metabolites biosynthesis, transport, and catabolism
PA5217	-4.5	Transcription
<i>foxI</i>	-4.1	Inorganic ion transport and metabolism

Since the expression levels of most iron acquisition related genes (e.g., *pvdA*, *pvdS* and *fpvA*) were downregulated after exposure to 100 μ M NO donor, the effect of NO on pyoverdine production was further investigated. Pyoverdine production was reduced by 25% after adding 100 μ M NO donor for 15 min (Figure 3.1). The effect of NO treatment did not appear to be dependent on the time of exposure, since a 15 min exposure showed a similar reduction in pyoverdine production as for 30 min exposure. Based on these results, it was hypothesized that NO induced repression of iron uptake systems may contribute to biofilm dispersal.

Figure 3.1. NO inhibits pyoverdine production. Biofilms were grown in multiwell plates for 6 h and treated with 100 μ M spermine NONOate (SP-NO) for 15 or 30 min. Relative fluorescence units (RFU) of pyoverdine were calculated by normalizing the pyoverdine fluorescence with the culture density as measured at OD₆₀₀. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples (*, $P < 0.1$).

3.3.2 Iron overrides NO induced dispersal independent of NO scavenging pathways

According to the RNA-Seq data and pyoverdine production results, it was hypothesized that NO induced biofilm dispersal through inhibition of iron uptake systems. To explore this hypothesis, the impact of the addition of exogenous iron on NO induced dispersal was explored. Biofilms were first treated with different concentrations of ferrous iron and NO simultaneously for 30 min. After treatment with 100 μ M of the NO donor alone for 30 min, as expected, more than 80% of biofilms were dispersed (Figure 3.2A). In contrast, when ferrous iron was added to biofilms at the same time as NO, the dispersal response appeared to be inhibited in an iron dose-dependent manner (Figure

3.2A), with only 27% of the biofilms dispersed after 30 min in the presence of 100 μM FeSO_4 . Further in the presence of iron, the biomass of biofilm not treated with NO also appeared to increase compared to biofilms that had not received iron or NO. These results suggested two possibilities, (i) that iron may interfere with NO sensing and the induction of dispersal, or (ii) that iron may affect the dispersal process further downstream in the regulatory cascade. To address this, biofilms were first exposed to NO alone for 15 min, which should allow enough time to induce the onset of dispersal events, before iron, in the ferric or ferrous form, was added to the cultures. Even when iron was added 15 min after NO, the biofilm biomass was found to increase in the presence of iron, with 100 μM FeSO_4 resulting in a 4.9, 4.7 and 3.6 fold increase after 15, 30 or 60 min, respectively (Figure 3.2B), which suggested that the effect of iron on the dynamics of dispersal was not dependent on its presence during NO release, NO sensing and the onset of dispersal. Further, similar increases in biofilm biomass were found after cells were exposed to 100 μM FeCl_3 (Figure 3.2B), indicating that this phenotype was not dependent on the iron oxidation-state (i.e. Fe^{+2} vs Fe^{+3}).

To further investigate if the inhibitory effect of iron on dispersal could be related to scavenging of NO, the scavenger PTIO was added instead of iron into cultures that had been treated with NO for 15 min in the same experimental system as described above. In the presence of 200 μM PTIO, the biofilm biomass of NO treated cultures appeared to be increased by 1.9 and 1.7 fold, after 15 and 30 min PTIO exposure, respectively, however the biofilm biomass was still lower in cultures treated with PTIO and NO compared to cultures with PTIO alone, indicating that some dispersal had occurred (Figure 3.2C). Further,

after 60 min, the biofilm biomass of PTIO treated cultures did not appear to continue to increase beyond the biomass of untreated control biofilms as was observed with iron. Because of the redox activity of iron, its potential direct impact on NO availability was assessed amperometrically using NO specific electrodes and a solution of 100 μ M SP-NO in buffer in the presence of 100 μ M FeCl₃, FeSO₄ or 200 μ M PTIO (Figure 3.2D). In the absence of any iron or scavenger, the amount of NO liberated from NO donor reached a steady state of more than 4 μ M within 15 min. The subsequent addition of 100 μ M FeSO₄ and 200 μ M PTIO caused a dramatic reduction of NO within 5 min, 97% and 98% lower, respectively. Interestingly, the amount of NO in solution in the presence of FeSO₄ recovered over time and achieved similar levels as the control after exposed to iron for about 15 min. In contrast, 100 μ M FeCl₃ only showed a slight reduction in NO concentration, 8% and 15% after 15 and 30 min, respectively. Taken together, exposure to PTIO did not induce biofilm formation of NO treated cells after 60 min (Figure 3.2C), while the addition of ferrous iron under the same conditions continuously induced hyper biofilm formation even after 60 min, although both PTIO and ferrous showed a similar reversible impact on the release of NO. Moreover, although NO exhibited completely different release profiles in the presence of FeCl₃ and FeSO₄, the biomass of NO treated biofilms was significantly increased in a similar fashion for both ferrous and ferric iron. These results suggest that iron overrides the NO induced dispersal response and this effect was independent of NO scavenging.

Figure 3.2. Iron overrides NO induced biofilm dispersal. (A) *P. aeruginosa* biofilms were grown in multiwell plates for 6 h and treated with 100 μM SP-NO and different concentrations of FeSO₄ simultaneously for 30 min before quantifying the biofilm biomass by CV staining. (B) and (C) biofilms were grown in multiwell plates for 6 h and treated with 100 μM SP-NO for 15 min. Then, biofilms were treated with 100 μM FeCl₃, FeSO₄ (B) or PTIO (C) for 15, 30 or 60 min before CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated control samples (*, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (D) Effect of FeCl₃, FeSO₄ and PTIO on NO release. NO levels in solution liberated from 100 μM SP-NO were measured amperometrically in the presence of 100 μM FeCl₃, 100 μM FeSO₄ and 200 μM PTIO, which were added 15 min after adding SP-NO as indicated by the arrow.

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

To investigate whether iron supplementation increases cell growth, Colony Forming Units (CFUs) of *P. aeruginosa* cells which have been previously incubated in multiwell plates and subsequently exposed to iron have been determined. After adding iron (Figure 3.3A) for 15, 30 or 60 min, the CFUs did not increase, suggesting that iron does not promote cell growth under these conditions. Additionally, the effect of NO on cell growth was tested. The cell numbers did not decrease in the presence of NO after 15, 30 or 60 min, demonstrating that NO was non-toxic at the concentrations used in this study (Figure 3.3B). Subsequent experiments were performed with FeCl₃ to avoid issues of the short-term loss of NO after adding FeSO₄.

Further, in the above batch culture multiwell plate biofilm dispersal assay in section 3.3.2, the NO donor 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 reattachment of the suspended cells containing both dispersed and planktonic cells. To discriminate between these two possibilities, iron was added into the wells only containing the suspended cells or the wells only containing the remaining biofilms (after suspended cells were removed and replaced with fresh medium).

Figure 3.3 Iron overrides NO induced biofilm dispersal via mainly promoting rapid attachment of suspended cells. Effect of Iron (A) and NO (B) on *P. aeruginosa* cell growth was tested. *P. aeruginosa* biofilms were grown in multiwell plates for 6 h and subsequently, supernatants containing planktonic cells were collected and transferred to 50 ml falcon tubes. Bacterial cells were incubated in the absence and presence of 100 μ M FeCl_3 (A) or SP-NO (B) for 15, 30 and 60 min and Colony Forming Units (CFUs) were calculated. Error bars indicate standard deviations ($n = 9$). *P. aeruginosa* biofilms were grown in multiwell plates for 6 h and subsequently dispersed by 100 μ M NO donor for 15 min. Subsequently, supernatants containing planktonic cells and dispersed cells were transferred to a new empty multiwell plate and fresh M9 medium was added into the original plate with remaining biofilms. Cultures including supernatants (C) and remaining biofilms (D) were then treated with 100 μ M FeCl_3 for 15, 30 and 60 min before CV staining. Error bars indicate standard deviations ($n = 6$). Asterisks indicate statistically significant differences compared to untreated control samples or between different samples (**, $P < 0.01$, ***, $P < 0.001$; ****, $P < 0.0001$).

The suspended cells attached quickly to a new, biofilm-free surface (Figure 3.3C). These cells showed an increase of biofilm biomass of 2.4, 3.2 and 6.2 fold after adding 100 μM FeCl_3 for 15, 30 and 60 min, respectively. In the presence of NO, the amount of biofilm was dramatically reduced compared to untreated cells, suggesting NO prevents the attachment of the suspended cells. When iron was added to NO treated cells, the suspended cells reattached and the biofilm biomass increased by 9.1, 8.8 and 4.1 fold after 15, 30 and 60 min, respectively. This suggests that iron promoted the fast attachment of planktonic cells regardless of the presence of NO. In contrast, the addition of iron did not enhance the biomass of NO treated biofilms (Figure 3.3D). Overall, the above results indicate that iron facilitates rapid biofilm formation of NO treated biofilms mainly by promoting the rapid attachment of planktonic and dispersed cells rather than through accelerating the growth of cells already within the remaining biofilms.

3.3.4 Iron and zinc both promote rapid attachment of planktonic cells

In order to explore whether the rapid attachment of planktonic cells was a specific response to iron, *P. aeruginosa* cells were incubated for 6 h and were exposed to different metals including iron, zinc, copper and manganese for 30 min. It was found that zinc also promoted cell attachment, as biofilm biomass was increased by 1.5 fold in the presence of 100 μM ZnCl_2 . In the presence of 1 and 10 μM CuSO_4 , biofilm biomass was increased by 1.4 fold. However, 100 μM and 1 mM CuSO_4 seemed to be toxic as the biofilm biomass was reduced by 74% and 82%, respectively. It was further hypothesized that reactive oxygen species (ROS) generated by those metals may be responsible for this effect and hence the effect of different concentrations of hydrogen peroxide (H_2O_2) on

biofilm formation was investigated. H_2O_2 at 1 mM seemed to be toxic as it reduced 71% biofilms. There was no significant difference in biofilm biomass between the untreated and samples treated with 100 nM to 100 μM H_2O_2 (Figure 3.4A), suggesting that ROS generated by these metals may not be responsible for the rapid attachment of planktonic cells.

Figure 3.4. Both iron and zinc promote rapid attachment of planktonic cells. *P. aeruginosa* biofilms were grown in multiwell plates for 6 h and treated with 100 nM, 1, 10, 100 μM and 1 mM FeCl_3 , ZnCl_2 , CuSO_4 , MnCl_2 and H_2O_2 for 30 min before CV staining (A). Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples (*, $P < 0.1$; ***, $P < 0.001$; ****, $P < 0.0001$). Effect of zinc (B) on *P. aeruginosa* cell growth was tested. *P. aeruginosa* biofilms were grown in multiwell plates for 6 h and subsequently supernatants containing planktonic cells were collected and transferred to 50 ml falcon tubes. Bacterial cells were incubated in the absence and presence of 100 μM ZnCl_2 for 15, 30 and 60 min. After that, CFUs of all bacterial cultures were calculated. Error bars indicate standard deviations ($n = 9$). Asterisks indicate statistically significant between different samples (*, $P < 0.1$).

As shown above, iron did not induce cell growth of *P. aeruginosa* (Figure 3.3A). After adding zinc for 15, 30 or 60 min (Figure 3.4B), CFUs of bacteria cells did not increase, suggesting that zinc does not promote cell growth in the current condition.

3.3.5 *psl* is required for iron and zinc induced fast attachment of planktonic cells

PSL, PEL and alginate are three of the main exopolysaccharides that play important roles in biofilm development and antibiotic resistance in *P. aeruginosa*. High levels of iron (50 and 100 μ M) have been recently reported to promote biofilm formation in *P. aeruginosa* via elevating PSL production (108). To determine if iron and zinc facilitated rapid attachment of planktonic cells through inducing the biosynthesis of those exopolysaccharides, the polysaccharide deficient mutants including *P. aeruginosa* Δpel , Δpsl and Δalg were tested (Figure 3.5A and B). Approximately 1.3 and 2.0 fold increases in biofilm biomass were observed when *P. aeruginosa* wild type was exposed to 100 μ M $FeCl_3$ and $ZnCl_2$, respectively. Iron and Zinc also accelerated the growth of Δpel biofilms by 1.3 and 1.5 fold. The Δalg mutant did not grow well in the current experimental conditions and its OD₆₀₀ was as low as 0.01 after 6 h incubation (Figure 3.5C and D). The Δpsl mutant grew well as planktonic cells but failed to form biofilms (Figure 3.5A and B), suggesting that PSL is important for biofilm formation in these experimental conditions. Interestingly, no attachment of the Δpsl planktonic cells was found in the presence of 100 μ M $FeCl_3$ or $ZnCl_2$ (Figure 3.5A and B).

Similar results were observed when cells were exposed to iron and zinc salts for 15 or 60 min (Figure 3.6). The results presented above suggest that PSL is

required for the iron and zinc induced rapid attachment of planktonic cells, although it remains to be determined if these metals induce the production of PSL to enhance attachment.

Figure 3.5. Iron and Zinc do not promote rapid attachment of planktonic cells of the *psl* mutant. Biofilms of *P. aeruginosa* wild type (WT) and mutant strains Δpel , Δpsl and Δalg were grown in multiwell plates for 6 h and treated with 100 μM FeCl₃ (A) or ZnCl₂ (B) for 30 min before CV staining. The number of planktonic cells treated with 100 μM FeCl₃ (C) or ZnCl₂ (D) was quantified by measurement of OD 600 nm. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated control samples (*, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Figure 3.6. Iron and zinc fail to promote rapid attachment of planktonic cells of the *psl* mutant. Biofilms of *P. aeruginosa* wild type (WT) and mutant strains Δpel , ΔpsI and Δalg were grown in multiwell plates for 6 h and treated with 100 μM FeCl_3 for 15 (A) or 60 min (B) or treated with 100 μM ZnCl_2 for 15 (C) or 60 min (D) before CV staining. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples (*, $P < 0.1$; **, $P < 0.01$; ****, $P < 0.0001$).

Further, to investigate whether iron and NO influence biofilm development through 1) modulating c-di-GMP with downstream effects on PSL or 2) by directly controlling PSL production or its release from the cells, PSL of *P. aeruginosa* biofilms before and after iron or NO treatment were quantified. After adding 100 μM FeCl_3 for 30 min, biofilm biomass was increased by 1.2 fold (Figure 3.7B) and PSL production increased by 2.7 fold (Figure 3.7C). These results showed that 100 μM FeCl_3 promoted PSL production and induced cell attachment.

Figure 3.7. Iron induces PSL production. *P. aeruginosa* biofilms grown in multiwell plate batch cultures for 6 h and subsequently left untreated or treated with 100 μ M FeCl_3 or 100 μ M NO donor for 30 min were stained with syto9 and TRITC-HHA where biofilms appear green and PSL stains red. Images of untreated, iron or NO treated biofilms were acquired by CLSM (A). 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 μ m. Biofilm (B) and PSL (C) quantification was performed using IMARIS. Error bars indicate standard deviations (n = 9). Asterisks indicate statistically significant differences compared to untreated control samples (**, $P < 0.01$; ****, $P < 0.0001$).

In contrast, treatment with 100 μ M NO donor for 30 min resulted in 79% biofilm dispersal (Figure 3.7B) with no change in PSL production (Figure 3.7C). Confocal images showed that PSL could still be visualized on biofilm surface after NO treatment (Figure 3.7A). This suggests that NO influences biofilm development through modulating c-di-GMP and likely has an indirect role in controlling PSL production.

3.3.6 Biofilms formed in the presence of iron can be dispersed by NO

NO disperses *P. aeruginosa* biofilms through the modulation of c-di-GMP (26), which has been reported to regulate PSL production at both transcriptional and translation levels (23). The results presented above indicate that iron overrides NO induced dispersal through promoting PSL-mediated rapid attachment of planktonic cells. To determine whether NO can disperse iron induced attached cells, 6 h cells of *P. aeruginosa* were exposed to 100 μ M FeCl₃ for 30 min and then were treated with NO over a time course of 15 to 60 min (Figure 3.8A).

After treatment with 100 μ M SP-NO for 15 min, 81% biofilms were dispersed in the absence of iron and 82% biofilms were detached in the presence of iron. When the NO treatment time was increased to 30 min, 78% and 77% of biofilms were dispersed in the absence and presence of iron, respectively, suggesting that NO can still disperse those freshly attached cells that were pre-exposed to iron. However, after adding NO for 60 min, 91% biofilms were removed, while the biomass of biofilms that had been pre-exposed to iron were only reduced by 22%. A more detailed, dynamic experiment showing the changes of biomass of 100 μ M FeSO₄ treated biofilms after exposure to NO within 60 min is shown in Figure 3.8B. It is clear that in the presence of iron, biofilms can still be dispersed by NO as the biofilm biomass decreased to 51%,

42%, and 32% of untreated biofilms biomass after 5, 10 and 15 min, respectively. However, the biofilm biomass slightly increased to 46%, 57%, 58%, 84% and 91% of untreated biofilm biomass after 20, 25, 30, 45 and 60 min. Taken together these results reveal that biofilms that are formed in the presence of iron can still be dispersed by NO, but that the dispersed cells will reattach in the presence of iron, most likely as a consequence of NO depletion over time.

Figure 3.8. Biofilms formed in the presence of iron can be dispersed by NO. *P. aeruginosa* biofilms were grown in multiwell plates for 6 h and treated with 100 μ M FeCl₃ for 30 min. Biofilms were then treated with 100 μ M SP-NO for 15, 30 or 60 min before CV staining (A). 6 h biofilms were treated with 100 μ M FeSO₄ for 30 min. Biofilms were then treated with 100 μ M SP-NO for 5, 10, 15, 20, 25, 30, 45 and 60 min before CV staining (B). Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples (****, $P < 0.0001$).

3.3.7 Iron-limited biofilms become more sensitive to NO

Because NO induces a decrease in pyoverdine production and that the presence of iron induces reattachment of dispersed cells, this leads to the possibility that depleting iron by the use of a chelator may enhance or extend the dispersal effect of NO. To investigate whether iron depletion enhanced or interfered with NO induced dispersal, biofilms were treated with NO in the presence or absence of the iron chelator, 2,2'-Bipyridine (Bipy) (Figure 3.9).

Figure 3.9 Iron-limited biofilms become more sensitive to NO. Biofilms grown in multiwell plates for 6 h were treated with 20 μ M SP-NO, 200 μ M Bipy and the combination of 20 μ M SP-NO and 200 μ M 2,2'-Bipyridine (Bipy) for 15, 30 and 60 min. Biofilm biomass was quantified by CV staining (OD₅₅₀). Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples or between different samples (*, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

After 15 min, 40% of the biofilms were dispersed by 20 μ M SP- NO while 65% of the biofilms were removed by the combined administration of 200 μ M Bipy and 20 μ M SP-NO. Treatment with Bipy and SP-NO for 30 min reduced the biofilms by 64%, which was greater than 20 μ M SP-NO alone, which reduced the biofilms by 34%. These results indicate that iron depletion can potentiate NO induced biofilm dispersal. Treatment with Bipy and SP-NO for 60 min reduced the biofilms by 41%, which was also greater than 20 μ M SP-NO alone (10% reduction). After treatment for 60 min, NO or combined administration of NO and Bipy did not further enhance biofilm dispersal compared to 15 and 30 min. This is probably because that in this system planktonic cells and dispersed cells started to reattach when the NO becomes depleted.

3.4 Discussion

3.4.1 The interplay between iron and NO in controlling biofilm development

As shown in section 3.3.1, the expression levels of most pyoverdine and pyochelin biosynthesis genes as well as iron acquisition related genes were lower in NO treated biofilms and dispersed cells compared to untreated cells, suggesting that iron uptake systems were inhibited by the addition of NO. In this work, it was further demonstrated that NO exposure reduced pyoverdine production of *P. aeruginosa*, which confirms previously published data (26). NO may impact the expression of iron uptake systems via the modulation of c-di-GMP. It has been reported that expression levels of iron uptake genes (*pvdA*, *pvdS*, *fpvA*, etc.) and pyoverdine production were lower in YhjH dispersed cells (1). In those experiments, dispersal was induced by lowering c-di-GMP through

the expression of a plasmid-encoded YhjH phosphodiesterase (PDE) in the presence of arabinose (171). In addition, overexpression of a c-di-GMP degrading enzyme (the PDE PA2133) inhibited pyoverdine production in *P. aeruginosa* (121). It is also noted that the Gac/Rsm system regulates siderophore production through modulation of c-di-GMP (121). Thus, it is clear that NO is able to affect pyoverdine synthesis via modulating c-di-GMP levels. Considering the fact that iron is an environmental signal to control biofilm formation (33, 108, 109), it was hypothesized that NO induces biofilm dispersal through inhibition of the iron uptake system. To explore this hypothesis, the effect of the exogenous addition of iron on NO induced dispersal was further explored. Ferrous iron and NO were added simultaneously into *P. aeruginosa* biofilms and it was found that iron overrode NO induced dispersal. NO can reversibly bind to ferric and ferrous iron but particularly rapidly and tightly to the latter (170). The binding rate of NO to ferrous heme is about 10^7 - 10^8 M⁻¹ s⁻¹ while the dissociation rate is as slow as less than 10^{-5} s⁻¹ (170). The tight binding causes a problem for NO as a signaling molecule. The binding rate of NO to ferric heme is about 10^7 M⁻¹ s⁻¹, whereas the off rate for the ferric-NO complex could be 10^4 to 10^8 times higher than the off rate of ferrous iron-NO complex (170). The reversible binding of NO to ferrous heme iron causes the activation of guanylate cyclase and inhibition of cytochrome activity while NO binding to ferric heme iron inhibits catalase production (170). In this study, the inhibition of iron on NO induced dispersal was unlikely to be due to the simple chemical interaction between NO and iron since ferric iron did not significantly reduce NO levels, despite inducing a hyperbiofilm phenotype. Furthermore, addition of the NO scavenger PTIO did not induce hyper biofilm formation.

The role of iron in reducing the NO mediated dispersal response was further found to be through the recruitment of planktonic cells back into the biofilms rather than enhancing growth of the existing biofilms. As described above, if biofilms were treated with NO and subsequently exposed to iron, biofilm biomass decreased due to the dispersing effect of NO and increased as iron promoted the fast attachment of dispersed cells and planktonic cells (Figure 3.2B). If biofilms were treated with iron and subsequently exposed to NO, the freshly formed biofilms can still be dispersed once NO was added (Figure 3.8). The same amount of iron and NO was used in those two experiments, while the only difference is that the order of adding iron and NO was reversed. This suggests that NO and iron may have antagonistic effects in biofilm development and those two factors may compete for the same regulatory pathway. It is therefore hypothesized that an oscillating biofilm formation could be observed when biofilms encounter sufficient and sustained amounts of iron and NO.

It was further demonstrated that *psl*, which is responsible for production of the primary biofilm matrix exopolysaccharide PSL, was required for iron-induced, fast attachment of planktonic cells. Similar results were recently reported, showing that high iron (50 and 100 μM FeCl_3) promoted PSL production and thus induced biofilm formation (108). In this study, it was also found that high iron (100 μM FeCl_3) induced PSL production (Figure 3.7). These results along with previous study support the hypothesis that high iron promotes biofilm formation or rapid attachment of planktonic cells via increasing the production of PSL. In *P. aeruginosa*, it is clear that NO disperses biofilms via modulating c-di-GMP which is a second messenger that regulates PSL production at both

the transcriptional and translation levels (23), although the direct link between NO and PSL has not been reported yet. Based on the above results, iron and NO may influence biofilm development through 1) modulating c-di-GMP with downstream effect on PSL or 2) by directly controlling PSL production or its release from the cells. However, the previous study has already indicated that c-di-GMP was not involved in iron-induced PSL production since the addition of iron had no impact on c-di-GMP levels (108). In addition to c-di-GMP, another main pathway in *P. aeruginosa* that drives the transition between biofilms and planktonic cells is the acylated homoserine lactone (AHL) based QS system. A previous study has demonstrated that high iron promoted PSL production through 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 (124-126). Therefore, it is suggested 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 was found to have no impact on PSL amount since most PSL still remained on surface after NO treatment although NO dispersed a big portion of PAO1 biofilms (Figure 3.7). NO is known to disperse biofilms through reducing c-di-GMP concentrations and requires the periplasmic protease LapG (167), which can cleave the protein adhesin CdrA off the cell surface (177). CdrA has been shown to either cross-links PSL polysaccharide polymers and/or tethers the cells to the PSL polysaccharide (166). Overall, it is suggested 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 bacteria cells and PSL, finally resulting in biofilm dispersal. Taken together, both iron and NO are controlling biofilm development, which are directly or indirectly linked to PSL.

Therefore, in the current study, it is suggested that iron and NO may control PSL through modulating QS and c-di-GMP, respectively. The results from this work have been used to modify a model that described in Chapter 1 (Figure 1.5), which links the regulatory pathway for iron, and NO based biofilm development and dispersal (Figure 3.10).

Figure 3.10. Model depicting the link between NO, c-di-GMP, iron, QS and biofilm development. This figure is modified from Figure 1.5 (Schematic adapted from (121, 122)). Blue lines highlight the new connections found or validated in this work. Black lines indicate previously described regulatory pathways.

3.4.2 Combined treatments of NO and iron chelators to 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 (130), while in the cystic fibrosis (CF) lung, the iron concentration varies from 2 to 130 μM (131-135). Therefore, the presence of iron could significantly impact the efficacy of NO when applied in industrial and clinical settings. As mentioned in Chapter 1, a range of iron-chelating compounds for biofilm control has been studied previously. In this study, one iron chelator, Bipy, was used in combination with NO and it was observed that NO treated biofilms were more sensitive to iron limitation and dispersed to a greater degree than when iron was not limiting. It was also reported that the combination of NO, iron chelator and tobramycin efficiently reduces the survival of the *P. aeruginosa* dispersed cells (1). Therefore, simultaneous treatments of biofilms with NO, iron chelator and biocides could be a powerful way to remove and kill biofilms in iron-rich environments. In summary, this chapter has linked two important environmental signals (iron and NO) with their roles in biofilm development and offered new perspective for improving the use of NO in biofilm control strategies.

Chapter Four: Nitric oxide Mediated Induction of Dispersal in *Pseudomonas aeruginosa* Biofilms Is Inhibited by Flavohemoglobin Production and Is Enhanced by Imidazole

This chapter has been reformatted from published data: **Zhu X, Oh HS, Ng YCB, Tang PYP, Barraud N, Rice SA. 2018.** Nitric oxide-mediated induction of dispersal in *Pseudomonas aeruginosa* biofilms is inhibited by flavohemoglobin production and is enhanced by imidazole. *Antimicrobial Agents and Chemotherapy* **62**:e01832-01817.

4.1 Introduction

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 (8, 9). The increased resistance of biofilms to antimicrobials, which can lead to the failure of treatment strategies to control them, is the cause of many problems in industrial and clinical settings. In the food industry, biofilms limit the run length of manufacturing plants, cause product spoilage and safety problems (178). In drinking water distribution systems, biofilms can cause corrosion or increase the risk of releasing microbial pathogens in the water (179). 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 (CF) patients and wound infection in severe-burn victims (15). Hence, it is of great interest to study biofilms with the aim of inhibiting biofilm formation.

Before the identification of nitric oxide (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 (37). 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 (180). 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 (181-184). For example, *P. aeruginosa* can detoxify NO by using either a NO reductase, NOR (42), or a flavohemoglobin, Fhp (42, 47). Fhp activity requires oxygen and this 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) (43), while Fhp is regulated by FhpR (47), which is homologous to the non-heme iron cofactor protein NorR capable of sensing NO in *Escherichia coli* (48). 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 (185), the expression of which is regulated by the Fe-S cluster regulator NsrR (186). 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 remain present at non-toxic levels (187, 188). In several bacterial species, the molecular mechanisms of NO mediated dispersal have been shown to involve bis-(3'-5')-cyclic dimeric guanosine monophosphate (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 protein domain, NosP, involved in the regulation of biofilm formation and dispersal via a multi-component phosphorelay system (63).

While the exogenous addition of a single dose of NO can disperse a significant portion of biofilms, it was recently found that multiple dosing of NO treatment does not improve biofilm dispersal (86). 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 (86). In this study, we hypothesized that after a first exposure to NO, a subpopulation of biofilm cells has an altered signaling pathway and become insensitive to NO. We investigated the response of *P. aeruginosa* biofilm cells to successive NO treatments and show that pre-exposure of biofilms to very low concentrations of NO inhibit 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 showed that this impaired dispersal can be restored by combined treatments with imidazole, a well-known antifungal drug that blocks the NO dioxygenase activity

of flavohemoglobin. Our findings offer a new perspective on reducing NO compound dosage and improving the use of NO as biofilm control strategies.

4.2 Materials and Methods

4.2.1 Bacteria and growth conditions

P. aeruginosa PAO1 wild type (WT) (145), PDO300, which is a *mucA22* derivative of PAO1 constructed by allelic exchange (189), and an isogenic Δ *mucA* mutant strain (190) were used in this study. *P. aeruginosa* PAO1 transposon mutant strains containing a transposon Tn5-derived insertion element (Tc^R) in key genes involved in NO detoxification were also used, that were obtained from the University of Washington *P. aeruginosa* mutant library (supported by grant # NIH P30 DK089507): Δ *fhp* (PA2664), strain PW5458 *fhp*-F06::*ISlacZ*/hah; Δ *fhpR* (PA2665), strain PW5460 *fhpR*-H11::*ISphoA*/hah; Δ *norB* (PA0524), strains PW1961 *norB*-A04::*ISphoA*/hah and PW1962 *norB*-A11::*ISphoA*/hah; Δ *norC* (PA0523), strains PW1959 *norC*-B02::*ISlacZ*/hah and PW1960 *norC*-H04::*ISphoA*/hah; Δ *hmgA* (PA2009), strain PW4489 *hmgA*-C03::*ISphoA*/hah; Δ *hpd* (PA0865), strains PW2577 *hpd*-H01::*ISlacZ*/hah and PW2578 *hpd*-H02::*ISlacZ*/hah; Δ *asrA* (PA0779), strains PW2411 *asrA*-A07::*ISlacZ*/hah, PW2413 *asrA*-G11::*ISlacZ*/hah and PW2414 *asrA*-C03::*ISlacZ*/hah; and Δ PA3697, strains PW7256 PA3697-H04::*ISlacZ*/hah, PW7257 PA3697-C03::*ISlacZ*/hah and PW7258 PA3697-D09::*ISphoA*/hah (191). For experiments involving the transposon mutants, the isogenic wild type strain MPAO1 was used (192); no difference in dispersal behaviour or response to NO has been observed between strains PAO1 and MPAO1. 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 cells for experiments.

4.2.2 NO donor compounds

The NO donor compound (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazene-1,2-diolate, spermine NONOate (SP-NO), was obtained from Cayman Chemical. SP-NO spontaneously dissociates to liberate two moles of NO per mole of the parent compound with a half-life of 39 min and 230 min at 37°C and 22-25°C, respectively (pH 7.4) (193, 194). 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.

4.2.3 Biofilm dispersal assays

Biofilms were grown as previously described (174) with some modifications. Briefly, overnight cultures of *P. aeruginosa* PAO1 wild type and mutants were diluted 200 fold to an OD₆₀₀ of 0.003 in M9 minimal medium (containing 9 mM NaCl, 22 mM KH₂PO₄, 48 mM Na₂HPO₄, 19 mM NH₄Cl, 2 mM MgSO₄, 100 µM CaCl₂ and 0.4% glucose, pH 7.0), which was freshly prepared for every experiment. One ml 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 into 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, biofilm biomass was analyzed by crystal violet (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 MilliQ water. Stained biofilms were washed twice with 1 ml PBS and the remaining CV was dissolved in 1 ml of absolute ethanol. 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 stained with the LIVE/ DEAD BacLight Bacterial Viability Kit reagents (Molecular Probes, Inc.) according to the manufacture's procedure. One microliter of each of the two components were mixed thoroughly in one ml of PBS, 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, biofilms were treated with and without 100 μ M 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 $\Delta mucA$ 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 MilliQ water at different concentrations. Biofilms that had been pretreated with 10 μ M SP-NO after 3 h growth (6 h biofilms), were then treated with 5 mM imidazole for 15 min prior to addition of 100 μ M SP-NO (15 min) 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 μ M SP-NO. Biofilm biomass was quantified by CV staining as described above.

4.2.4 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 μ M and calibrated by using MAHMA NONOate (Cayman Chemical) as the NO donors.

NO dioxygenase (NOD) activity of purified Fhp was also assessed amperometrically by following a protocol previously described by Gardner and

Gardner (1995). NO was released from 100 μ M SP-NO in a NOD activity buffer solution consisting of 100 mM Na_3PO_4 , pH 7.0, 0.3 mM EDTA, 100 μ M NADPH (Cayman Chemical), and 1 μ M 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 into NOD activity buffer in the presence of FAD, NADPH and NO. Final concentrations of Fhp and imidazole were 5 nM and 5 mM, respectively.

4.2.5 Generation of complemented strain

A *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* (Table B.1) using Pfu polymerase (Thermo Fisher Scientific) and ligating it into *Eco*RI and *Bam*HI (New England Biolabs) double cut plasmid pUCP22. The resultant plasmids were transformed in the *E. coli* donor strain DH5 α and transformed to the transposon mutant Δfhp (PW5458 *fhp*-F06::IS*lacZ*/hah).

4.2.6 Quantitative Real-Time Reverse-Transcription PCR (qRT-PCR)

cDNA was reverse transcribed from 0.5 μ g of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) as per manufacturer protocols. 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*. Primers are listed in Table B.1. 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 $2^{-\Delta\Delta C_t}$ method (196).

4.2.7 Fhp protein purification

Cloning and protein expression were carried out at the Protein Production Platform (PPP) at Nanyang Technological University (NTU). Targets of *fhp* (1175 bp), truncated *fhp* without flavin-containing domain (422 bp) and truncated *fhp* without heme-binding domain (716 bp) were amplified using primers Fhp-f7852/Fhp-r7876, Fhp-f7852/Fhp-r7880 and Fhp-f7854/Fhp-r7877 (Table B.1). All cloning was performed in 96 well systems using ligation-independent cloning (LIC) technology in *E. coli* (197, 198). Histidine-tagged protein production was induced with IPTG and analyzed on SDS-PAGE gels. Clones with acceptable levels of expression were selected for large-scale expression and purification. During purification steps, cell lysates were loaded on IMAC columns (1 mL Ni-NTA Superflow (Qiagen)) and were washed. His-tag 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 including 20 mM HEPES (pH 7.5), 300 mM NaCl, 10% (v/v) glycerol and 2 mM TCEP. The final protein batch was then aliquoted into smaller fractions, frozen in liquid nitrogen and stored at -80°C.

4.2.8 Statistical analysis

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

4.3 Results

4.3.1 NO pretreated biofilms show impaired dispersal

Previously, it was found that multiple doses of NO did not significantly increase biofilm dispersal (86). To better understand this phenomenon, the response of biofilm cells to NO was investigated by using a batch culture multiwell plate biofilm dispersal assay (174). Biofilms, with no pretreatment, grown for a total of 6 h 15 including a dispersal treatment (15 min) were reduced by 88% after exposure to a dispersing dose, 100 μ M, of the NO donor spermine NONOate (SP-NO) compared to control non-dispersed biofilms as seen with CV staining (Figure 4.1 A) (Schematics of experiments are shown in Figure B.1A a and c). 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 biofilm dead cells (Figure B.2), showing that the 100 μ M SP-NO is not affecting viability of the biofilm cells. This is comparable to the results from CV staining, where the 6 h biofilms were reduced by 88% (Figure 4.1A). In this system, biofilms that were pretreated with 10 μ M SP-NO (non-dispersing dose) for 3 h before inducing dispersal with 100 μ M SP-NO for 15 min had similar biomass with and without the second NO treatment (Figures 4.1A) (Schematics of experiments are shown in Figure B.1A 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 μ M SP-NO) was altered after pre-exposure to non-dispersing concentrations of NO (10 μ M SP-NO). However, pretreated biofilms were dispersed when the concentration of the second dose of NO was increased to 200 or 500 μ M (Figure 4.1B). This suggests that the factor responsible for the pretreatment effect could be titrated away by increasing the effective concentration of NO.

Figure 4.1. Biofilms that were pretreated with a low dose of NO require increased concentrations of NO to induce dispersal. *P. aeruginosa* biofilms were grown in multiwell plate batch cultures for a total of 6 h 15, including or not a pretreatment with 10 μ M SP-NO at $t = 3$ h, and a dispersal treatment with 100 μ M (A), 200 μ M (B) or 500 μ M (B) spermine NONOate (SP-NO) at $t = 6$ h. At the end of the incubation, biofilm biomass was analyzed by crystal violet (CV) staining. Experimental details are shown in Figure B.1A. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples (**, $P < 0.01$; ****, $P < 0.0001$). Each image represents the CV stained biofilms.

Two strains that overproduce alginate were also tested using the pretreatment assay: PDO300, which is a *mucA22* derivative of PAO1 constructed by allelic exchange (189), and an isogenic Δ *mucA* mutant strain (190). Both strains could be dispersed by a single dose of 100 μ M NO, as was observed for the non-mucoid *P. aeruginosa* PAO1 strain. Biofilms of PDO300 and the Δ *mucA* mutant that were pre-exposed to 10 μ M SP-NO were also impaired in their dispersal response after a subsequent exposure to a second effective dose of NO (100 μ M SP-NO) (Figure 4.2), which is similar to the wild type PAO1 strain.

Figure 4.2. Biofilms of alginate-overproducing *P. aeruginosa* strains were also impaired in their dispersal response after a pretreatment with NO. Biofilms of *P. aeruginosa* wild type (WT), PDO300 and Δ *mucA* were grown in M9 minimal medium supplemented with 0.4% glucose and 2% (w/v) casamino acid in multiwell plate batch cultures for a total of 6 h 15, including or not a pretreatment with 10 μ M SP-NO at t = 3 h, and a dispersal treatment with 100 μ M SP-NO at t = 6 h. At the end of the incubation, biofilm biomass was analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated control samples (*, $P < 0.1$; **, $P < 0.01$; ****, $P < 0.0001$).

In the same system, exposure of biofilms formed by the wild type PAO1 strain to 10 μ M SP-NO did not prevent carbon or oxygen starvation induced dispersal (Figure 4.3A and B), suggesting that the pretreatment induces a NO specific response.

Figure 4.3. NO pretreated biofilms are dispersed by starvation and oxygen depletion. (A) *P. aeruginosa* biofilms were grown in multiwell plate batch cultures for a total of 6 h 30 including or not a pretreatment with 10 μ M SP-NO at t = 3 h and a dispersal treatment with fresh M9 medium in the presence (non-starvation) or absence (starvation) of 0.4% glucose at t = 6 h for 30 min. At the end of the incubation, biofilm biomass was analyzed by CV staining. (B) For oxygen depletion induced dispersal, after 6 h of incubation with fast agitation at 180 rpm, the shaking speed was maintained at 180 rpm or reduced to 60 rpm to reduce oxygen tension and the biofilms were incubated for a further 30 min before CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (***, P < 0.001; ****, P < 0.0001).

4.3.2 NO scavenging activity is cell associated and not secreted

Because the change in dispersal behaviour after pretreatment appeared to be specific to NO mediated dispersal and could be surpassed by using higher levels of the signal molecule, we then 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 here, and at a concentration of 100 μM of donor compound, the amount of NO in solution was found to reach a steady-state of 3.7 μM after 7 min (Figure 4.4A). 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 (Figure 4.4A). In contrast, the cell-free supernatant of pretreated cells did not have any impact on NO levels (Figure 4.4B), suggesting that the NO scavenging capacity was not due to secreted factors, but was associated with intracellular components. This was also supported by the fact replacing the supernatant of biofilm cultures did not alleviate the dispersal prevention effect of pretreatment with NO (Figure 4.4C). Taken together these results indicate that NO pretreatment induced the production of NO scavenging factors, which were cell-associated and not secreted.

Figure 4.4. Free NO is scavenged by NO pretreated *P. aeruginosa* cells rather than the NO pretreated cell-free supernatant. NO levels in solution liberated from 100 μ M SP-NO were measured amperometrically, in the absence or presence of *P. aeruginosa* cells that grown in multiwell plates for 3 h and treated or not with 10 μ M SP-NO for a further 3 h (A) or in the absence or presence of *P. aeruginosa* cell-free supernatant (B), which was grown in multiwell plates for 3 h and exposed to 10 μ M SP-NO, incubated for a further 3 h (6 h total) before collection.

Figure 4.4. (Cont'd) (C) Replacing the supernatant does not impact on NO pretreatment induced impaired dispersal. Biofilms were grown in multiwell plate batch cultures for 6 h. After 6 h, the biofilm culture supernatants were either replaced or not with fresh M9 medium in the presence or absence of 100 μ M SP-NO and the plates were incubated for a further 15 min, followed by assessment of total biofilm biomass by CV staining. For the pretreatment experiments, biofilms were grown for 3 h, exposed to 10 μ M SP-NO, and incubated for further 3 h incubation (6 h total) before replacing the supernatant and being exposed to 100 μ M SP-NO. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated samples (*, $P < 0.1$; ****, $P < 0.0001$).

4.3.3 *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 (45). To determine if these proteins may be involved in scavenging NO and preventing dispersal, transposon mutant strains including *P. aeruginosa* Δfhp , $\Delta norB$, $\Delta norC$, $\Delta hmgA$ and Δhpd were tested using the pretreatment assay. In contrast to all of the mutants tested (Figure B.3), the *P. aeruginosa* Δfhp mutant was readily dispersed in response to 100 μ M SP-NO despite pretreatment with 10 μ M SP-NO (Figures 4.5A). Complementation of the Δfhp mutant restored the wild type phenotype with regards to the dispersal defect. Further, even in the absence of pretreatment, the complemented strain showed enhanced resistance in to 100 μ M SP-NO induced dispersal compared to the wild

type (Figure 4.5A). 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.

Figure 4.5. *P. aeruginosa* Δfhp and $\Delta fhpR$ mutant strains are not affected by NO pretreatment. Biofilms of *P. aeruginosa* wild type (WT), transposon mutants Δfhp (A) and $\Delta fhpR$ (B) and the Δfhp strain complemented with pFhp plasmid (A) were grown in multiwell plate batch cultures for a total of 6 h 15, including or not a pretreatment with 10 μ M SP-NO at t = 3 h, and a dispersal treatment with 100 μ M SP-NO at t = 6 h. At the end of the incubation, biofilm biomass was analyzed by CV staining. Error bars indicate standard deviations (n = 3). 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.

Transcriptional activity of the *fhp* promoter is known to be dependent on the regulators FhpR, AsrA and PA3697 (47, 199). To determine which regulator of *fhp* may be involved in the dispersal impairment, transposon mutant strains $\Delta fhpR$, $\Delta asrA$ and PA3697 were tested. Results showed that NO pretreated biofilms formed by *P. aeruginosa* $\Delta fhpR$ also lost their defect in dispersal (Figure 4.5B). In contrast, the transposon mutants $\Delta asrA$ and PA3697 were not affected (Figure B.4). This suggests that *fhpR* may play a role in regulating the production of NO scavenging proteins after exposure to low levels of NO.

4.3.4 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 impaired of dispersal, we hypothesized that *fhp* may be upregulated after an exogenous pretreatment stimulation. 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 (Figure 4.6). Expression levels of *fhp* were also similar in 6 h planktonic cells and 6 h biofilms (data not shown). Treatment with 10 μ M 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 (Figure 4.6), suggesting that *fhp* expression does not only happen in planktonic cells but can also occur in biofilms, which could compromise biofilm control strategies that rely on using NO as a dispersing agent.

Figure 4.6. NO pretreatment induces the expression of *fhp*. *P. aeruginosa* PAO1 was grown in multiwell plate batch cultures for 3 h before being treated with or without 10 μ M SP-NO. After 15 min, mRNAs were extracted from biofilm and planktonic bacteria and *fhp* mRNA levels were quantified by real-time quantitative reverse transcription PCR (qRT-PCR) analysis. Data show fold changes in *fhp* transcript levels relative to untreated planktonic cells. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences between samples (**, $P < 0.01$; ***, $P < 0.001$).

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 (Figure 4.7A). 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 (200). 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 compared to 6 h biofilms (Figure 4.7B), although these changes were not statistically significant due to the big standard error. In this system, *fhp* was also induced in 12, 16 and 20 h unstimulated biofilms compared to 8 h biofilms (Figure 4.7C). 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.

Figure 4.7. *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 which allows for continuous increase in biofilm biomass over 20 h (B). (C) Time-course analysis of *fhp* mRNA levels in the 20 h biofilm system. Error bars indicate standard error (n = 3). Asterisks indicate statistically significant differences between samples (**, $P < 0.01$).

4.3.5 In older biofilms, inactivation of *fhp*, but not *fhpR*, leads to enhanced sensitivity towards 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 may be altered in older biofilms. The effects of 10 and 100 μ M SP-NO were tested on 20 h biofilms of *P. aeruginosa* wild type and isogenic Δfhp and $\Delta fhpR$ mutant strains. In this biofilm assay, a low dose of NO generated from 10 μ M SP-NO dispersed 86% of biofilm biomass in the *P. aeruginosa* Δfhp mutant, while only 10% and 26% of biofilms formed by *P. aeruginosa* wild type and $\Delta fhpR$ were dispersed by 10 μ M SP-NO (Figure 4.8A). In the presence of 100 μ M SP-NO, 90% of both wild type and Δfhp biofilms were removed and $\Delta fhpR$ biofilms were dispersed by about 61%. This suggests that 20 h biofilms of Δfhp were more sensitive to NO signals than wild type and $\Delta fhpR$ mutant strains. qRT-PCR analysis of 20 h wild type and $\Delta fhpR$ biofilms did not show any significant differences in *fhp* mRNA levels between these strains (Figure 4.8C), suggesting that *fhpR* was not necessary for the non-stimulated, endogenous induction of *fhp* in 20 h biofilms. However, in contrast to the wild type strain, Δfhp and $\Delta fhpR$ mutant strains still retained their dispersal capacity after pretreatment stimulation with an initial low dose of NO in the 20 h biofilms system (Figure 4.8B), which correlates with the results previously obtained with the 6 h biofilm system (Figure 4.1A)

Figure 4.8. In 20 h biofilms, inactivation of *fhp*, but not *fhpR*, leads to enhanced sensitivity towards NO mediated induction of dispersal. (A) The Δfhp mutant strain was more sensitive to NO mediated induction of dispersal compared to the wild type strain and $\Delta fhpR$ mutant in 20 h biofilms. Biofilms of *P. aeruginosa* wild type (WT), and transposon mutant strains Δfhp and $\Delta fhpR$ grown in multiwell plate batch cultures at room temperature for 20 h were treated with 10 μ M or 100 μ M SP-NO for 15 min before CV staining. Experimental details are shown in Figure B.1B. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples (****, $P < 0.0001$). (B) Δfhp and $\Delta fhpR$ 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* wild type (WT), and transposon mutant strains Δfhp and $\Delta fhpR$ were grown in multiwell plate batch cultures for a total of 20 h 15, including or not a pretreatment with 10 μ M SP-NO at $t = 10$ h, and a dispersal treatment with 100 μ M SP-NO at $t = 20$ h. Each image represents the stained biofilms.

Figure 4.8. (Cont'd) At the end of the incubation, biofilm biomass was analyzed by CV staining. Experimental details are shown in Figure B.1C. Error bars indicate standard deviations ($n = 3$). 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 $\Delta fhpR$ mutant. Error bars indicate standard error ($n = 3$). ns = No significant difference.

4.3.6 Imidazole blocks the NO pretreatment effect and enhances dispersal by inhibiting flavohemoglobin

Because the production of Fhp impaired NO mediated dispersal, it was hypothesized that inhibiting the NOD activity of Fhp could restore dispersal in pretreated biofilms. One such candidate inhibitor compound is imidazole, which has been previously reported to hinder the NOD activity of bacterial and fungal flavohemoglobins by coordinating and constraining their heme (201). 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 the NO specific electrodes and an appropriate buffer system to study NOD kinetics (201). As expected, the addition of 5 nM Fhp purified from *P. aeruginosa* dramatically reduced the amount of NO released from 100 μ M SP-NO (Fig 4.9A). In contrast, Fhp that had been treated with imidazole did not have any effect on the release of NO.

Figure 4.9. Imidazole can inhibit the NO dioxygenase (NOD) activity of Fhp and restore the dispersal response of *P. aeruginosa* biofilms pretreated with NO. (A) NO released from 100 μ M 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 μ M NADPH, and 1 μ M FAD at room temperature. Fhp (5 nM) either untreated or treated with imidazole (5 mM) were added 15 min after adding SP-NO. (B) NOD activity of purified Fhp and truncated Fhp. Fhp and truncated Fhp were added 15 min after adding SP-NO. (C) *P. aeruginosa* biofilms were grown in multiwell plate batch cultures for 6 h. Then, biofilms were treated or not with 5 mM imidazole (Im) for 15 min, after which time dispersal was induced with 100 μ M 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 μ M SP-NO, and incubated for a further 3 h incubation (6 h total) before being exposed imidazole and NO. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistically significant differences compared to untreated control samples (***, $P < 0.001$; ****, $P < 0.0001$). Images show the CV stained biofilms.

Furthermore, truncated Fhp proteins that lacked the flavin-binding or heme-binding domains both lost the ability to scavenge NO (Figure 4.9B). In the absence of imidazole, 6 h biofilms pretreated with a low dose of NO (10 μ M SP-NO) were impaired in their dispersal response to 100 μ M SP-NO as expected. However, the addition of 5 mM imidazole for 15 min prior to the final treatment with 100 μ M SP-NO restored the dispersal response, resulting in the detachment of 64% of biofilms (Figure 4.9C). 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.

To investigate whether the addition of imidazole can improve the dispersal response to NO in *P. aeruginosa*, biofilms were further investigated 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 μ M SP-NO) successively. It was found that combined treatments of imidazole (5 or 10 mM) and 10 μ M SP-NO failed to disperse 6 h *P. aeruginosa* biofilms (Figure 4.10A). In contrast, in 20 h biofilms, although 10 μ M SP-NO alone also had no impact on these biofilms, combined treatments of imidazole (5 and 10 mM) and 10 μ M SP-NO dispersed about 88% of these biofilms (Figure 4.10B).

Figure 4.10. 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 (Im) for 1 h prior to addition of 10 μ M SP-NO for 15 min. Error bars indicate standard deviations (n = 3). At the end of the incubation, biofilm biomass was analyzed by CV staining. Asterisks indicate statistically significant differences compared to untreated control samples (****, $P < 0.0001$). The images show the CV stained biofilms.

4.4 Discussion

4.4.1 An endogenous mechanism to limit biofilm dispersal

In this work, an experimental system was designed to test the effect of successive exposure to NO on biofilm dispersal. The previously observed attenuation of dispersal responses after multiple treatments with NO was also observed here, by first exposing biofilms to a low, non-dispersing concentration followed by NO concentrations that would normally disperse biofilms. By using this system, it was found that the pretreatment effect was due to a 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 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. This clarifies that, despite the observation that 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 (167), the oxygen and nutrient controlled dispersal pathways are independent of NO signaling, and 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^-) (47, 202) and this reaction is dependent on NADPH, FAD and oxygen (203). These results showed that the expression level of *fhp* was highly induced by a non-dispersing dose of NO (10 μM SP-NO) in both biofilms and planktonic cells. The transcriptomic data in Chapter 2 also showed that *fhp* was 105.3 fold higher in NO (100 μM SP-NO) treated compared to untreated biofilms (Table A.2) and 162.9 fold higher in dispersed cells compared to untreated planktonic cells (Table A.5). The induction of *fhp* after exposure to 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 (26). However, the experimental conditions in the previous studies were different, as biofilms were grown for five days and exposed to the NO donor sodium nitroprusside (SNP) 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 compared to 6 h biofilms.

In *P. aeruginosa*, NO could also be reduced to N₂O by NOR (204, 205), 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 (47). 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 (47). FhpR is a homologue of NorR in *E. coli*, which regulates production of flavorubredoxin (48, 185). It was previously reported that the *fhp* promoter is dependent on two additional regulators, AsrA and PA3697 (199), although neither appeared to be required in our assays for the pretreatment induced impaired dispersal (Figure B.4). 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 amongst prokaryotic and eukaryotic microorganisms (206). In *E. coli* and *Staphylococcus aureus*, the expression of genes encoding flavohemoglobins has been shown to be stimulated by NO and nitrosative stresses (129, 207). 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 (208, 209). 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 allow for its successful colonization of the host while other invading bacterial species are excluded (210, 211).

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 (27). 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. This may also explain why a subpopulation of biofilms always fails to NO induced dispersal. What remains to be determined is the local expression patterns of *fhp* in different parts of the biofilm and in particular, how this might correlate with different physiological states of the cells or the availability of electron donors or acceptors (e.g. oxygen). Future studies in our laboratory will investigate the dynamics and spatial localization of *fhp* expression during biofilm development and dispersal by creating a *fhp* reporter strain.

4.4.2 Combined treatments of 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 (212-215). Here, imidazole was found to block the NO pretreatment induced impairment of dispersal and 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 μ M SP-NO, while 6 h biofilms did not disperse to such low dose of NO. 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 capable of scavenging NO to some levels 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 (216-218). For example, sponges of the genera *Leucetta* and *Clathrina* are rich sources of imidazole alkaloids (219-221). In the future, it will be interesting to investigate the combined effect of low dose of NO and different imidazole derivatives with low toxicity and high bioavailability on biofilms formed by single- and multi-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 a 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 new perspective on improving the use of NO for biofilm control in both clinical and industrial settings.

Chapter Five: Summary and General Discussion

The exogenous addition of nitric oxide (NO) has been shown to induce dispersal of biofilms formed by a broad range of microorganisms via the modulation of intracellular bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) concentrations, a secondary messenger molecule in bacteria that controls the transition between planktonic cells and biofilms through stimulating exopolysaccharide synthesis, flagella and pili based motility (21, 22). It was recently shown that NosP is involved in the regulation of biofilm formation and dispersal via a multi-component phosphorelay system (63). However, despite the clear link between NO and the c-di-GMP signaling pathway, the direct NO sensor that can transmit the NO signal into intracellular changes and c-di-GMP levels has not been fully elucidated in *Pseudomonas aeruginosa*. Thus, the identification of a NO binding receptor and subsequent molecular pathway, including NosP, that are involved in the modulation of c-di-GMP, as well as the specific targets of gene regulation that are involved in NO induced dispersal remain to be elucidated. This pathway is of particular interest to better understand how bacteria perceive and respond to NO to control dispersal. For example, one of the key questions is whether the NO dispersal pathway operates in a single pathway that does not interact with other global regulatory systems or if it is integrated into other decision making processes in the cell. For example, in *Vibrio cholerae*, c-di-GMP based signaling pathway activates biofilm formation while another signaling pathway, quorum sensing (QS), represses biofilm formation (222). QS is a signaling process where bacteria respond to changes in population density or local diffusion constraints

through monitoring signal concentration and therefore changing gene expression (223). HapR, a major regulator of QS, represses biofilm formation in *V. cholerae* through controlling the transcription of 14 genes encoding proteins that synthesize and degrade c-di-GMP, resulting in increased c-di-GMP levels at low cell density and decreased c-di-GMP levels at high cell density (222). In *P. aeruginosa*, it is possible that the NO response is also integrated with other response mechanisms of bacteria to optimise dispersal in response to a number of environmental and physiological factors.

Additionally, determination of the molecular NO pathway can lead to the identification of additional genes or proteins that can be targeted to potentiate the NO signal to more comprehensively induce dispersal. Therefore, one main objective of this study was to search for new molecular targets that are potentially involved in NO induced dispersal. To achieve this goal, a global, non-targeted approach investigating the change in both transcriptome as well as sRNA expression was undertaken. In this study, transcriptomic profiles of untreated biofilms, planktonic cells, the biofilm cells that do not disperse after exposure to the NO donor spermine NONOate (SP-NO) and SP-NO dispersed cells were compared in the same system, which extends previous studies that have only investigated the untreated and NO treated biofilms that were pregrown in the tubing biofilm system for five days (27) or compared RNA profiles of untreated planktonic cells, biofilms and nitroprusside (SNP) dispersed cells that were grown in the microtiter plates (1).

As shown in Chapter 2, the RNA profile of the biofilm cells that failed to disperse after NO treatment and NO dispersed cells were first compared in the current study. Based on analysis of the top 20 genes that showed significant

differences in gene expression between those two types of cells, transposon mutants were selected from a saturated Tn library and screened for changes in NO induced dispersal. However, none of those mutants showed an altered response to NO under the conditions used here. One possible explanation for this observation is that the NO dispersal response is dependent on multiple genes or gene products and that no single gene, at least in the set identified and tested here, is essential for dispersal. It is also possible that genes that showed a lower response in terms of change in gene expression (e.g. outside of the top 20 tested here), could play a major role in the dispersal response. Thus, further pathway analysis, for example, the Gene Set Enrichment Analysis (GSEA), and protein-protein interaction of suspected proteins by using methods such as the pull-down assay will be conducted to identify genes that contribute to the NO resistance.

In addition to the roles of various genes in controlling biofilm development and dispersal, it is increasingly recognised that bacteria rely on small RNAs (sRNA) to modulate phenotypes. In *P. aeruginosa*, RsmY and RsmZ have been found to be involved in NO induced dispersal as they were downregulated in NO dispersed cells compared to planktonic cells (1). RsmW was previously shown to be upregulated in *P. aeruginosa* PAO1 biofilms compared to planktonic cells (82) and SrbA has been reported to be important for biofilm formation of *P. aeruginosa* PA14 (77). However, although more than 200 sRNAs have been characterized so far in *P. aeruginosa*, the role of sRNA in biofilm dispersal is still unclear. Therefore, a comprehensive analysis of the role of sRNAs in controlling the NO induced dispersal response was undertaken here. In this study, the sRNA sequencing data highlighted several sRNA candidates that

may be involved in NO induced dispersal. The list of sRNA genes that were altered in expression upon NO addition is listed in Chapter 2. Further experiments including making deletion mutants of sRNA genes will be conducted to study the roles of sRNA candidates. Apart from studying the role of sRNA in NO induced dispersal, it will be also interesting to investigate the role of sRNA in biofilm development that controlled by another environmental signal, iron. As mentioned above, a pair of sRNAs, PrrF1 and PrrF2, which are under control of Fur, are posttranscriptional regulators that induce upregulation of genes involved in iron storage, oxidative stress resistance and intermediary metabolism (75, 106). However, a previous study showed that those two sRNAs were not required for biofilm formation in the flow cell system (97). High iron has been shown to induce the production of PSL (108). PSL and PEL have been reported to positively regulate the expression of RsmY and RsmZ in the presence of high c-di-GMP (122). Hence, high iron may promote biofilm formation through inducing PSL and therefore improve the expression of sRNA RsmY and RsmZ. In the future, to investigate the role of regulatory sRNA that link iron and biofilm development, it would be interesting to build the sRNA profiles of biofilms with and without iron treatment using RNA-Seq. Moreover, searching for sRNAs that respond to both NO and iron may help to clarify the link between NO and iron in biofilm development.

5.1 Cells exhibit different NO responsive profiles in the current system from previous studies

mRNA-Seq in this thesis has yielded different results from previous studies. For example, this study has shown a high induction (over 100 fold) of *fhp* after

exposure to the dispersing dose of NO (100 μ M SP-NO) or a low, non-dispersing dose of NO (10 μ M SP-NO). These results were surprising as the previous study revealed a 1.8 fold downregulation of *fhp* in NO treated biofilms compared to untreated biofilms (26) or no significant difference of the expression of *fhp* between untreated planktonic cells, biofilms or NO dispersed cells (1). Different experimental conditions including incubation time and oxygen levels may explain the huge difference in *fhp* expression between those studies. Biofilms in this study were grown with shaking and a shorter incubation time (3 h or 6 h) before quantifying the *fhp* expression level, while in previous studies, biofilms were incubated under static conditions and grown for 24 h or 5 d. It is possible that in more mature biofilms, *fhp* exhibits different expression and regulation profiles, which has been supported by the observation that *fhp* expression was higher in 20 h biofilms compared to 6 h biofilms. Alternatively, the high expression of Fhp may only occur in biofilms under aerobic condition, for example, in the current system, since Fhp has an oxygen-dependent dioxygenase function that converts NO to nitrate (NO_3^-) (47, 202, 203). This was also supported by the observation that NOR which could reduce NO to N_2O under anaerobic or low-oxygen conditions (47) was not involved in the impairment of NO induced dispersal and neither *norB* nor *norC* were induced or inhibited after NO treatment. Interestingly, the expression of *norB* and *norC* was highly induced (over 100 fold) in NO or YhjH dispersed cells compared to untreated planktonic cells and biofilms (1). In another study, many genes involved in anaerobic metabolism, for example, *nirSMLJ*, encoding nitrite reductase activity and *norC* were induced in SNP treated biofilms. However, none of these genes showed significantly changed expression levels

after NO treatment in the current study. This finding has provided evidence supporting the hypothesis that the current biofilm system is under aerobic conditions, while biofilms in the previous studies were experiencing anaerobic conditions.

P. aeruginosa can grow and form biofilms under both aerobic and anaerobic conditions since it can utilize oxygen (aerobic) as well as nitrate, nitrite, and nitrous oxide (anaerobic) as terminal electron acceptors during respiration (224). The ability of *P. aeruginosa* to grow under anaerobic conditions enables this strain to form biofilms in the Cystic Fibrosis (CF) lung, where *P. aeruginosa* converts to a mucoid, alginate-overproducing phenotype (225, 226). In the CF, the iron concentration varies from 2 to 130 μM (131-135). Iron is an environmental cue that influences biofilm formation through modulating QS, effecting DNA release, enhancing production of the main exopolysaccharide PSL and other mechanisms (33, 108, 109). It has been shown that the increased airway iron is important to *P. aeruginosa* persistence in CF (227). Synthetic iron-binding compounds have been found to interfere with *P. aeruginosa* biofilm formation, especially under anaerobic conditions (228). In this thesis, the roles of iron and NO on biofilm development and their interplay have been investigated but the study was conducted on aerobic biofilms. Hence, it is still unclear how do *P. aeruginosa* biofilms behave in response to NO and iron under anaerobic or low-oxygen condition. In the future, it would be interesting to study the role of NO, iron and their responsive signaling pathways in *P. aeruginosa* under anaerobic or low-oxygen condition.

5.2 Two challenges in using NO as a biofilm control strategy: the production of Fhp induced by NO itself and the presence of iron in environment

A recent study has shown that multiple doses of NO treatment does not improve biofilm dispersal (86). This could be due to NO induced defense or that the biofilm contains sub-populations, e.g. persisters or dormant cells, which do not respond to NO. These cells may lack NO sensing proteins or produce high amounts NO scavenging enzymes, for example, NO reductase. Therefore, another goal of this thesis focused on understanding why the biofilm does not disperse completely and why additional doses of NO do not disperse the biofilm further. As shown in Chapter 4, after the first exposure to NO, FhpR can sense NO to trigger production of the flavohemoglobin Fhp and inhibit subsequent dispersal responses to NO. This is the first study to identify a novel role of the NO scavenging flavohemoglobin Fhp, where Fhp is induced in an NO dependent fashion in *P. aeruginosa* biofilms and impairs the NO induced dispersal. Fortunately, imidazole compounds, which inhibit NOD function by coordinating heme iron and “fit” within the heme pocket since they have bulky aromatic substituents (212-215), were further found to block the NO pretreatment induced impairment of dispersal and work synergistically with NO to disperse biofilms at very low concentrations of NO donors. A next step of this study would be to search for naturally occurring and synthetic derivatives of imidazole or other compounds with low toxicity and high bioavailability that can inhibit Fhp and improve the dispersing effect of NO.

The RNA-Seq data has revealed a large overlap in genes downregulated upon exposure to NO and those expressed under iron-limiting conditions. This is

supported by previous reports showing that some pyoverdine and pyochelin synthesis related genes (*pvdG*, *pvdH*, *pchB*, *pchC*, *pchD*, *pchE*, *pchG*, *pchF* and *fpvA*) were downregulated in NO or YhjH dispersed cells (1). Iron is known to effect formation through QS, effecting DNA release, enhancing production of the main exopolysaccharide PSL and other mechanisms (33, 108, 109). Moreover, *P. aeruginosa* $\Delta pvdA$, $\Delta pvdS$ and $\Delta fpvA$ mutants, defective in genes that are important for pyoverdine synthesis, signaling and uptake (97, 172), form thin layers of biofilms instead of mushroom-like structure in the flow cell system (97), suggesting an important role of pyoverdine in biofilm formation. The involvement of iron has been supported by the study showing that iron chelator (e.g., EDTA, lactoferrin, deferoxamine and deferasirox) induce biofilm dispersal (136, 141, 142).

In this study it was hypothesized that iron serves as an environmental signal that interferes with NO induced dispersal. Alternatively, NO may disperse biofilms through controlling iron or iron uptake system. Hence, one goal of this study was to investigate the interplay between two important environmental signals, iron and NO, and their roles in biofilm development. As shown in Chapter 3, iron could override NO induced biofilm dispersal through promoting the rapid attachment of planktonic cells, which is a PSL-dependent phenotype. In a previous study, high iron (50 and 100 μM FeCl_3) has been shown to promote PSL production, which has been validated in this study as well, and thus induced biofilm formation, although this effect may be mediated through the modulation of QS rather than c-di-GMP (108). Moreover, NO dispersed freshly attached cells that were induced by iron. NO disperses biofilms via modulating c-di-GMP that regulates PSL production at both the transcriptional

and translation levels (23), although the direct link between NO and PSL has not been reported yet. In the current study, NO was found to have no impact on PSL amount since most PSL still remained on surface after NO treatment although NO dispersed a big portion of PAO1 biofilms (Figure 3.7). Therefore, it is suggested 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 bacteria cells and PSL, finally resulting in biofilm dispersal. Taken together, both iron and NO are controlling biofilm development, which are directly or indirectly linked to PSL.

The presence of iron in environment is potentially an obstacle when NO is to be used as biofilm control strategy in industrial and clinical contexts. In natural environments, bacteria can encounter varying levels of available iron. For example, in addition the CF lung as mentioned above, iron is present in wastewater at an average of 9 μM (130). Therefore, the presence of iron could significantly impact the efficacy of NO when applied in industrial and clinical settings. Results presented in Chapter 3 have demonstrated that NO treated biofilms were more sensitive to iron limitation by adding the iron chelator 2,2'-Bipyridine (Bipy) and dispersed to a greater degree than when iron was not limiting. This finding suggests that simultaneous treatments of biofilms with NO, iron chelator and biocides will be a powerful way in removing and killing biofilms in iron-rich environment.

5.3 Potential targets that improve NO induced biofilm dispersal

This thesis has uncovered potential targets in NO induced biofilm dispersal. RNA-Seq data have highlighted several sRNA that may play regulatory roles in

NO induced dispersal. Understanding the roles of those sRNA and their downstream regulatory pathway have implication in improving biofilm dispersal, for example, blocking or promoting sRNA that positively or negatively regulates biofilm formation or dispersal. Moreover, the interplay between iron and NO has indicated that interacted signaling pathway rather than one single signaling pathway are involved in biofilm development. Thus, blocking one signaling pathway may potentiate the effects on another regulatory pathway. This has been further supported by observation that NO treated biofilms were more sensitive to iron stress induced by Bipy. However, Bipy has not been approved by the US Food and Drug Administration (FDA) and therefore, other compounds, for example the FDA approved iron chelators including deferoxamine and deferasirox, could be used instead of Bipy in subsequent studies. Another finding in this study was that imidazole can improve NO induced dispersal through inhibiting the function of Fhp. However, imidazole is toxic and the concentrations (5 and 10 mM) used in this study were very high. Imidazole has a moderate oral toxicity, with a LD₅₀ in rats of 960-970 mg/kg body weight (229). Hence, it is necessary to look for imidazole derivatives with low toxicity and high bioavailability. In addition to imidazole, other compounds that have similar structure that can bind the heme pocket could be used as candidates as well. Since iron chelators can bind to iron while the imidazole has a high affinity to the heme pocket, it would be interesting to investigate the effect of combined administration of NO, iron chelator and imidazole on biofilms formed by single- and multi-species. It has been reported that the combination of NO, iron chelator and tobramycin efficiently reduced the survival of the dispersed cells (1). Therefore, simultaneous treatment of

biofilms with imidazole its derivatives and an iron chelator in combination with NO could be a powerful way to disperse biofilms formed in an iron-rich environment.

5.4 Summary

In summary, this is the first study that compares transcriptomic profiles of both messenger RNA and small, non-coding RNA of untreated biofilms, planktonic cells, remaining biofilms after NO treatment and NO dispersed cells in the same system. The remaining biofilms and NO dispersed cells exhibited different transcriptomic profiles, which may help to understand the reason of resistance of remaining biofilms. Furthermore, the sRNA sequencing results have highlighted several new targets that may be involved in NO induced dispersal, which provides possible targets for biofilm control. Moreover, in this study, it has been found that NO induced dispersal in *P. aeruginosa* is not a single signaling pathway as both NO and iron serve as signaling molecules in controlling biofilms through interacted signaling pathways. NO disperses *P. aeruginosa* biofilms through modulating c-di-GMP, which controls PSL at both transcriptional and translation level (23), while the effect of iron in overriding NO induced dispersal via promoting fast cell attachment, which is a PSL dependent phenotype and it is likely linked to QS. The two main signaling relays that drive the transition between biofilms and planktonic cells, QS and c-di-GMP, both control pyoverdine production but through different pathways. In this study, pyoverdine production and iron acquisition systems were found to be inhibited after exposure to NO. Furthermore, this study has found that FhpR can sense NO to trigger production of the flavohemoglobin Fhp and inhibit

subsequent dispersal responses to NO, which answers the question that why multiple dosing of NO treatment does not improve biofilm dispersal (86). It is likely that Fhp is under the control of the ferric uptake regulator *fur* in *P. aeruginosa*, since its homologue, Hmp, was regulated by Fur in *Salmonella typhimurium* (128). The interplay between NO, iron, Fur, pyoverdine, QS, c-di-GMP and their links to the transition between biofilms and planktonic cells have been clearly described in Figure 5.1. Overall, the present thesis addressed the mechanisms of NO induced biofilm dispersal in *P. aeruginosa* and offered new perspective for improving the use of NO in biofilm control strategies, for example, using imidazole to inhibit Fhp and using iron chelators to control iron concentrations.

Figure 5.1. Model depicting the link between NO, c-di-GMP, iron, QS and biofilm development. This figure is modified from Figure 1.5 and Figure 3.10 (Schematic adapted from (121, 122)). Black lines indicate previously described regulatory pathways. Blue lines highlight the new connections found or validated in Chapter 3. Pink lines indicate the link that has been validated in Chapter 4. Green line shows the uncertain link between Fur and Fhp.

Appendix A

Table A.1. List of bacterial strains used in Chapter 2.

Strain	Remark	References
PAO1	Wild type	(145)
MPAO1	Wild type	University of Washington <i>Pseudomonas aeruginosa</i> mutant library
Δ PA4155	PW8041 PA4155-F11::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ femR	PW4349 femR-G05::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ femI	PW4351 femI-F10::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA1923	PW4368 PA1923-D11::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA1925	PW4370 PA1925-F04::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA0433	PW1791 PA0433-A09::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA4837	PW9134 PA4837-F06::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA3600	PW7113 PA3600-C02::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ pvdQ	PW1693 pvdQ-D07::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA5059	PW9496 PA5059-E09::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ pyrQ	PW10392 pyrQ-A04::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA4834	PW 9128 PA4834-H06::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA4835	PW9130 PA4835-B12::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA4836	PW9132 PA4836-D03::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA0434	PW1793 PA0434-D04::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA2034	PW4518 PA2034-H04::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ cyoC	PW3393 cyoC-E11::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ tadD	PW8244 tadD-E11::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library

Table A.1. List of bacterial strains used in Chapter 2 (Cont'd).

Strain	Remark	References
Δ PA1539	PW3775 PA1539-G08::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA3009	PW6040 PA3009-A08::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ hutU	PW9563 hutU-B01::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ lpdV	PW4823 lpdV-G10::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA4683	PW8884 PA4683-F02::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA2555	PW5289 PA2555-E08::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ liuA	PW4494 liuA-F04::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA2224	PW4788 PA2224-C11::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA3052	PW6117 PA3052-D09::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA0840	PW2529 PA0840-G04::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ map	PW7184 map-B01::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ nuoA	PW5409 nuoA-D06::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ PA1964	PW4426 PA1964-A10::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ bkdA1	PW4817 bkdA1-A11::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ bkdA2	PW4820 bkdA2-E08::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ bkdR	PW4816 bkdR-B08::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ liuR	PW4496 liuR-G06::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library
Δ bfrB	PW6980 bfrB-A04::ISlacZ/hah	University of Washington <i>P. aeruginosa</i> mutant library

Table A.2. Top 20 upregulated and downregulated in NO treated and remaining biofilms compared to untreated biofilms genes (with a P-value cut-off of 0.05).

	Gene	Fold change	COG
1	PA5106	238.6	Nucleotide transport and metabolism / General function prediction only
2	<i>hutU</i>	107.6	Amino acid transport and metabolism
3	<i>metE</i>	107.6	Amino acid transport and metabolism
4	<i>flhP</i>	105.3	Energy production and conversion
5	<i>bkdA1/bkdA2</i>	90.6	Energy production and conversion
6	<i>bfrB</i>	71.8	Inorganic ion transport and metabolism
7	PA2662	57.1	Inorganic ion transport and metabolism
	<i>ppyR</i>		Inorganic ion transport and metabolism
8	<i>liuA</i>	44.2	
9	<i>lpdV</i>	43.9	Lipid metabolism
10	<i>hpd</i>	31.3	Energy production and conversion
11	PA5099	30.8	Amino acid transport and metabolism / General function prediction only
12	PA3532	30.1	Nucleotide transport and metabolism
13	<i>liuR</i>	28.6	
14	PA5104	17.7	Function unknown
	<i>hutC</i>		Transcription
15	PA4658	16.1	Function unknown
16	PA3690	15.1	Inorganic ion transport and metabolism
17	<i>liuB</i>	14.8	Lipid metabolism
18	PA2557	13.9	Lipid metabolism / Secondary metabolites biosynthesis, transport, and catabolism
19	PA2691	12.8	Energy production and conversion
20	PA0840	12.7	Energy production and conversion
	Gene	Fold change	COG
1	PA4063	-32.8	
2	<i>femR</i>	-25.2	Inorganic ion transport and metabolism / Signal transduction mechanisms
	<i>femI</i>	-25.2	Transcription
3	PA4837	-22.3	Inorganic ion transport and metabolism
4	<i>fumC1</i>	-13.8	Energy production and conversion
	PA4471		
5	PA4129	-12.9	Function unknown
	PA4130		Inorganic ion transport and metabolism
6	<i>ilvH</i>	-12.2	Amino acid transport and metabolism

Table A.2. Top 20 upregulated and downregulated genes (with a P-value cut-off of 0.05) in NO treated and remaining biofilms compared to untreated biofilms (Cont'd).

	Gene	Fold change	COG
7	<i>tonB1</i>	-11.4	Cell envelope biogenesis, outer membrane
8	<i>phuS</i>	-11.1	Inorganic ion transport and metabolism
9	<i>cbpD</i>	-10.4	Function unknown
10	<i>cyoA</i>	-10	Energy production and conversion
11	PA4469	-9.8	
12	PA4156	-9.5	Inorganic ion transport and metabolism
13	<i>pvdQ</i>	-9.1	General function prediction only
	<i>phzC1</i>		Amino acid transport and metabolism
14	<i>phzD1</i>	-9	Secondary metabolites biosynthesis, transport, and catabolism
	<i>phzE1</i>		Amino acid transport and metabolism / Coenzyme metabolism
15	PA0433	-8.7	
16	PA2069	-8.6	Posttranslational modification, protein turnover, chaperones
17	<i>pfeR</i>	-8.4	Signal transduction mechanisms / Transcription
	<i>pfeS</i>		Signal transduction mechanisms
	<i>mexI</i>		Defense mechanisms
18	<i>opmD</i>	-8.1	Cell envelope biogenesis, outer membrane / Intracellular trafficking and secretion
19	<i>ureC</i>	-8.1	Amino acid transport and metabolism
20	<i>phzA1</i>	-8	

Table A.3. Top 20 upregulated and downregulated genes in NO dispersed cells compared to untreated biofilms (with a P-value cut-off of 0.05).

	Gene	Fold change	COG
1	PA5106	78.8	Nucleotide transport and metabolism / General function prediction only
2	<i>flp</i>	71.8	Energy production and conversion
3	<i>bkdA1/bkdA2</i>	55.8	Energy production and conversion
4	PA2662	55.2	Inorganic ion transport and metabolism
	<i>ppyR</i>		
5	<i>metE</i>	48	Amino acid transport and metabolism
6	<i>flp</i>	23.5	
7	<i>bfrB</i>	20.4	Inorganic ion transport and metabolism
8	PA4702	18.3	
9	PA2779	17	
10	<i>pra</i>	15.5	

Table A.3. Top 20 upregulated and downregulated genes in NO dispersed cells compared to untreated biofilms (with a P-value cut-off of 0.05) (Cont'd).

	Gene	Fold change	COG
11	PA0788	15.5	Cell envelope biogenesis, outer membrane
12	<i>hpd</i>	15	Amino acid transport and metabolism / General function prediction only
13	<i>czcR</i>	12.9	Signal transduction mechanisms / Transcription
13	<i>czcS</i>	12.9	Signal transduction mechanisms
14	<i>hutU</i>	12.3	Amino acid transport and metabolism
15	PA1414	11.4	
16	PA3690	11.2	Inorganic ion transport and metabolism
17	PA0201	10.2	General function prediction only
18	<i>agtB</i>	9.8	Amino acid transport and metabolism
19	PA1874	9.3	
20	PA4027	9.1	Amino acid transport and metabolism / Signal transduction mechanisms
	Gene	Fold change	COG
1	PA4063	-8.4	
2	PA3720	-7.4	
3	PA4515	-7.4	Function unknown
4	PA2790	-6.2	
5	<i>mdcA</i>	-5.7	Lipid metabolism
6	PA0209	-5.7	Coenzyme metabolism
	<i>pyrE</i>	-5.6	Nucleotide transport and metabolism
7	PA4064	-5.6	Defense mechanisms
8	<i>desB</i>	-5.2	Lipid metabolism
8	PA4889	-5.2	Energy production and conversion
9	PA1343	-5.2	
10	<i>rho</i>	-5.1	Transcription
11	PA3119	-5.1	Coenzyme metabolism
12	PA1768	-4.9	Posttranslational modification, protein turnover, chaperones
13	<i>ahpF</i>	-4.9	Posttranslational modification, protein turnover, chaperones
14	PA3009	-4.7	
15	PA1964	-4.5	General function prediction only
16	PA3431	-4.4	Cell envelope biogenesis, outer membrane
	PA3432		General function prediction only
17	<i>edd</i>	-4.3	Amino acid transport and metabolism / Carbohydrate transport and metabolism
18	<i>senC</i>	-4.3	General function prediction only
19	<i>pbpA</i>	-4.1	Cell envelope biogenesis, outer membrane
20	PA5225	-4	Function unknown

Table A.4. Top 20 upregulated and downregulated genes in NO dispersed cells compared to remaining biofilms (with a P-value cut-off of 0.05).

	Gene	Fold change	COG
1	PA4155	15.2	Energy production and conversion
2	<i>femR</i>	10.9	Inorganic ion transport and metabolism /
	<i>femI</i>		Signal transduction mechanisms
3	PA1808	10.2	Transcription
4	PA1914	10.1	General function prediction only
	PA1922	9.3	Inorganic ion transport and metabolism
5	PA1923	9.3	Coenzyme metabolism
	PA1924		Intracellular trafficking and secretion
	PA1925		Function unknown
6	PA0433	8.9	
7	PA4837	7.8	Inorganic ion transport and metabolism
8	PA1878	7.4	General function prediction only
9	PA3600/PA3601	7.3	Translation, ribosomal structure and biogenesis
10	PA0781	6.9	Inorganic ion transport and metabolism
11	<i>pvdQ</i>	6.7	General function prediction only
12	<i>coaB</i>	6.5	
13	PA5059	6.4	Transcription
14	<i>pyrQ</i>	6.2	Nucleotide transport and metabolism
15	PA2366	6.2	Intracellular trafficking, secretion, and vesicular transport
	PA4834	6.2	Carbohydrate transport and metabolism /
16			Amino acid transport and metabolism /
	PA4835		General function prediction only
	PA4836		Function unknown
17	PA0434	6.1	Inorganic ion transport and metabolism
18	PA2033	6	Inorganic ion transport and metabolism
	PA2034		
19	<i>cyoC/cyoD</i>	6	Energy production and conversion
20	<i>tadC</i>	5.9	Cell motility and secretion / Intracellular trafficking and secretion
	<i>tadD</i>		Intracellular trafficking and secretion
	Gene	Fold change	COG
1	PA1539	-11.1	
2	PA3009	-9.4	
3	<i>hutU</i>	-8.8	Amino acid transport and metabolism
4	<i>lpdV</i>	-8.7	Energy production and conversion

Table A.4. Top 20 upregulated and downregulated genes in NO dispersed cells compared to remaining biofilms (with a P-value cut-off of 0.05) (Cont'd).

	Gene	Fold change	COG
5	PA4683	-6.8	
6	PA2555	-5.9	Lipid metabolism
7	<i>liuA</i>	-5.8	Lipid metabolism
8	<i>pyrE</i>	-5.7	Nucleotide transport and metabolism
9	<i>wzz</i>	-5.7	Cell envelope biogenesis, outer membrane
10	PA4658	-5.4	Function unknown
11	PA2224	-5.2	
12	PA3052	-5.1	
13	PA0840	-5.1	Energy production and conversion
14	<i>map</i>	-5.1	Translation, ribosomal structure and biogenesis
15	<i>nuoA</i>	-4.9	Energy production and conversion
16	PA1964	-4.9	General function prediction only
17	PA3532	-4.9	
18	PA0529	-4.8	Function unknown
19	<i>liuB</i>	-4.8	Lipid metabolism
20	PA5099	-4.7	Nucleotide transport and metabolism

Table A.5. Top 20 upregulated and downregulated genes in NO dispersed cells compared to untreated planktonic cells (with a P-value cut-off of 0.05).

	Gene	Fold change	COG
1	<i>flhP</i>	162.9	Energy production and conversion
2	<i>metE</i>	159.3	Amino acid transport and metabolism
3	PA5106	113.7	Nucleotide transport and metabolism / General function prediction only
4	PA2662 <i>ppyR</i>	106.2	Inorganic ion transport and metabolism
5	<i>bfrB</i>	76.7	Inorganic ion transport and metabolism
6	<i>oprH</i>	75.8	
7	<i>glpD</i>	53.1	Energy production and conversion
8	<i>bkdA1/bkdA2</i>	48.3	Energy production and conversion
9	<i>hutU</i>	25.1	Amino acid transport and metabolism
10	PA3417/PA3416	21.8	Energy production and conversion
11	PA4702	19.8	
12	<i>pra</i>	19	
13	<i>liuR</i>	17.1	Transcription
14	PA2024	16.8	Amino acid transport and metabolism

Table A.5. Top 20 upregulated and downregulated genes in NO dispersed cells compared to untreated planktonic cells (with a P-value cut-off of 0.05) (Cont'd).

	Gene	Fold change	COG
15	PA1414	15.3	
16	PA0788	15.1	Cell envelope biogenesis, outer membrane
17	PA4139	14.5	
18	PA5303	14	Translation, ribosomal structure and biogenesis
18	<i>dadA</i>	14	Amino acid transport and metabolism
19	PA0201	13.4	General function prediction only
20	PA3235	13.3	Function unknown
	Gene	Fold change	COG
1	<i>phzA1</i>	-7	
2	<i>htpG</i>	-6.7	Posttranslational modification, protein turnover, chaperones
3	PA4063	-6.3	
4	<i>phzA2</i>	-6.2	
5	<i>lasA</i>	-5.8	Cell envelope biogenesis, outer membrane
6	PA1768	-5.7	Posttranslational modification, protein turnover, chaperones
7	<i>pvdA</i>	-5.6	Secondary metabolites biosynthesis, transport, and catabolism
8	PA4798	-5.4	Function unknown
9	<i>pilH</i>	-5.4	Signal transduction mechanisms / Transcription
10	<i>pvdS</i>	-5.3	Transcription
11	<i>phzB1</i>	-5.3	
12	<i>mexG</i>	-5.2	Function unknown
13	PA5217	-4.5	Inorganic ion transport and metabolism
14	<i>pcrV</i>	-4.4	
15	<i>mexH</i>	-4.3	Cell envelope biogenesis, outer membrane
16	<i>foxI</i>	-4.1	Transcription
17	PA0020	-4	Function unknown

Table A.6. Top 20 upregulated and downregulated genes in untreated biofilms compared to untreated planktonic cells (with a P-value cut-off of 0.05).

	Gene	Fold change	COG
1	<i>glpD</i>	48.5	Energy production and conversion
2	<i>oprH</i>	35	
3	<i>desB</i>	19.2	Lipid metabolism
	PA4889		Energy production and conversion

Table A.6. Top 20 upregulated and downregulated genes in untreated biofilms compared to untreated planktonic cells (with a P-value cut-off of 0.05) (Cont'd).

	Gene	Fold change	COG
4	<i>mdcA</i> PA0209	9.9	Lipid metabolism Coenzyme metabolism
5	<i>phoP</i> <i>phoQ</i>	7.8	Signal transduction mechanisms / Transcription Signal transduction mechanisms
6	<i>glpK</i>	7.4	Energy production and conversion
7	PA4139	6.6	
8	<i>hdhA</i>	6.2	Energy production and conversion
9	<i>cmpX</i> <i>arnB</i> , <i>arnC</i> <i>arnA</i>	6.1	Cell envelope biogenesis, outer membrane Translation, ribosomal structure and biogenesis
10	<i>arnD</i> <i>arnT</i> <i>arnT</i> <i>arnF</i> PA3559	6	Carbohydrate transport and metabolism Cell envelope biogenesis, outer membrane
11	PA5303 <i>dadA</i>	5.9	Cell envelope biogenesis, outer membrane Translation, ribosomal structure and biogenesis Amino acid transport and metabolism
12	PA1053	5.3	Cell envelope biogenesis, outer membrane
13	PA1964	5	General function prediction only
14	PA2440/PA2441	4.8	
15	<i>capB</i>	4.6	Transcription
16	PA1112.1	4.5	
17	<i>spuA</i>	4.4	General function prediction only
18	<i>flgF</i>	4.3	Cell motility and secretion
19	<i>metR</i>	4.1	Transcription
	Gene	Fold change	COG
1	PA1036	-7.3	
2	PA5286	-6.4	Function unknown
3	PA2860	-6.3	
4	PA0926	-4.9	
5	PA2166	-4.6	
6	<i>htpG</i>	-4.6	Posttranslational modification, protein turnover, chaperones
7	PA2381	-4.5	
8	<i>corA</i>	-4.5	Inorganic ion transport and metabolism
9	<i>flp</i>	-4.2	
10	PA0622	-4.1	General function prediction only

Figure A.1. BfrB is not involved in NO induced dispersal. Biofilms of *P. aeruginosa* wild type (A) and the transposon mutant $\Delta bfrB$ (B) were grown in multiwell plate batch cultures for 6 h. At the end of the incubation, biofilms were exposed to 100 μ M spermine NONOate (SP-NO) for 15 min before crystal violet (CV) staining to quantify the amount of biofilm. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (***, P < 0.001; ****, P < 0.0001).”

Appendix B

Figure B.1. Schematics of experiments shown in Figure 4.1 (A), Figure 4.8A (B) and Figure 4.8B (C). The arrows indicate the time (after inoculation) and concentration of nitric oxide (NO) addition for different experiments. For all NO treatments at 6 or 20 h, biofilms were incubated for 15 min before being assessed for biofilm biomass by CV staining.

Figure B.2. Treatment with 100 μ M SP-NO disperses biofilms and does not impact viability of biofilm cells. *Pseudomonas aeruginosa* biofilms grown in multiwell plate batch cultures for 6 h and subsequently left untreated (A) or treated with 100 μ M SP-NO (B) for 15 min were stained with the LIVE/ DEAD BacLight Bacterial Viability Kit where live cells appear green and dead cells stain red. Images of untreated (A) and NO treated (B) biofilms were acquired by CLSM. 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 20 μ m. Biofilm quantification (C) was performed using IMARIS. Error bars indicate standard deviations (n = 6). Asterisks indicate statistically significant differences compared to untreated control samples (****, $P < 0.0001$). ns = No significant difference.

Figure B.3. NorB, NorC, HmgA and Hpd are not involved in the dispersal impairment. Biofilms of transposon mutant strains $\Delta norB$ PW1962 *norB*-A04::IS*phoA*/hah (A) and PW1961 *norB*-A11::IS*phoA*/hah (B), $\Delta norC$ PW1959 *norC*-B02::IS*lacZ*/hah (C), and PW1960 *norC*-H04::IS*phoA*/hah (D), $\Delta hmgA$ PW4489 *hmgA*-C03::IS*phoA*/hah (E), Δhpd PW2577 *hpd*-H01::IS*lacZ*/hah (F), and PW2578 *hpd*-H02::IS*lacZ*/hah (G) were grown in multiwell plate batch cultures for a total of 6 h 15, 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, biofilm biomass was analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (**, P < 0.01).

Figure B.4. *AsrA* and *PA3697* are not involved in the dispersal impairment. Biofilms of transposon mutants $\Delta asrA$ PW2411 *asrA*-A07::IS*lacZ*/hah (A), PW2413 *asrA*-G11::IS*lacZ*/hah (B), and PW2414 *asrA*-C03::IS*lacZ*/hah (C), $\Delta PA3697$ PW7256 *PA3697*-H04::IS*lacZ*/hah (D), PW7257 *PA3697*-C03::IS*lacZ*/hah (E) and PW7258 *PA3697*-D09::IS*phoA*/hah (F) were grown in multiwell plate batch cultures for a total of 6 h 15, 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, biofilm biomass was analyzed by CV staining. Error bars indicate standard deviations (n = 3). Asterisks indicate statistically significant differences compared to untreated samples (*, P < 0.1; ***, P < 0.001; ****, P < 0.0001).

Table B.1. Primers used in Chapter 4.

Primer name	Sequence (5' – 3')
<i>fhp_for</i>	ATTTTCGACCTCTTTGCAGTC
<i>fhp_rev</i>	CTCCTTTTTTCCGAAAAAGGG
<i>fhp_RT_for</i>	AATTACCTGCATGACCGGGTC
<i>fhp_RT_rev</i>	CGGAAACAGATCCAGCTCGT
<i>rpoD_RT_for</i>	CCTGCGCCTGGTGATTT
<i>rpoD_RT_rev</i>	GTGGCGTAGGTGGAGAACTT
Fhp-f7852	TACTTCCAATCCATGTTGTCCAATGCCCAACGTGC
Fhp-r7876	TATCCACCTTTACTGTCAGGCGTCCAGCGCGGCGG
Fhp-r7880	TATCCACCTTTACTGTCAATACACCGACTCCTCGGCC
Fhp-f7854	TACTTCCAATCCATGGATGGCGGCTGGCGCGG
Fhp-r7877	TATCCACCTTTACTGTCACGGACCGAAGAACTCGTAG

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