Novel nitric oxide donors for the dispersal and control of *Pseudomonas aeruginosa* biofilms

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NANYANG
TECHNOLOGICAL
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**NOVEL NITRIC OXIDE DONORS FOR THE DISPERSAL AND
CONTROL OF *PSEUDOMONAS AERUGINOSA* BIOFILMS**

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CONTROL OF *PSEUDOMONAS AERUGINOSA* BIOFILMS**

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Doctor of Philosophy

2017

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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Abbreviations

CdGMP	Cyclic-di-GMP
CF	Cystic fibrosis
CV	Crystal violet
Col	Colistin
DGC	Diguanylate cyclase
EPS	Extracellular polymeric substance
IMIP	Imipenem
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MIC	Minimum inhibitory concentration
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
NO	Nitric oxide
NONOates	Diazeniumdiolates
PAS	Pas-Arnt-Sim
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
QS	Quorum sensing
S150	Spermine NONOate

Abstract

The use of nitric oxide (NO) represents a novel strategy for the control of biofilms by inducing biofilm dispersal and promoting the eradication of susceptible, dispersed cells by antibiotic treatment. NO, as a free radical, is highly reactive and its production and metabolism is tightly regulated under physiological conditions. In addition, due to the involvement of NO in various pathological processes, several classes of NO donors with varying pharmacological properties have been developed for different treatments. In the case of biofilm control, two main classes of NO donor are typically used to induce biofilm dispersal, sodium nitroprusside (SNP) and diazeniumdiolates (NONOates). The latter of which generally have short half-lives. In the first study, an alternative class of NO donors, furoxans, which can have an extended duration of NO-release, was evaluated as a biofilm dispersing agent using *P. aeruginosa* biofilms. It was shown that a furoxan with a shorter half-life, LL4254, could induce dispersal to a similar extent as a control NONOate. Furoxans with longer half-lives, LL4212 and LL4216, could also induce biofilm dispersal, but only when used at higher concentrations. As LL4212 and LL4216 were later found to affect the growth of *P. aeruginosa*, they were considered unsuitable for use as biofilm dispersing agents. Thus, the study indicated that secondary effects of NO donor compounds were important considerations for their development as biofilm control agents.

In the second study, the application of cephalosporin-linked diazeniumdiolate NO donors, which are stable until activation by bacterial specific β -lactamases present within biofilms, were evaluated. The study laid the groundwork for the assessment of these compounds *in vivo*, as factors influencing NO release from these compounds were evaluated. While the compounds were effective in dispersing *P. aeruginosa* biofilms *in vitro*, their activity was limited *in vivo*, likely due to differences in experimental conditions in the two systems. Preliminary assessment of one lead compound, DEACP, in a mouse implant model of biofilm infection also suggested that dispersal may lead to poor

prognosis, as higher bacterial counts were retrieved from spleen samples of mice in NO-treated groups as a consequence of dispersal. Further studies would be required for optimization of the use of these compounds *in vivo*. In addition, antibiotic treatment is recommended for clearance of dispersed bacteria to reduce incidences of sepsis due to dispersed cells.

In the last study, genes and proteins that may be involved in NO sensing and NO induced dispersal were tested for their response to NO treatment. Two purified proteins, DipA and RbdA, were not found to act as sensors of NO, and are likely to be involved in the dispersal process through other mechanisms. Using a batch microplate system for biofilm dispersal, transposon mutants of several genes previously shown to be defective in dispersal in flow systems, were unexpectedly found to be unaffected in NO induced dispersal, indicating that there may be genetic redundancies and that the response to NO and that NO induced dispersal may be additionally regulated by the growth conditions.

1. Literature Review

1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a bacterium that is ubiquitous in the environment, and is commonly found in natural water bodies, soil and as part of the natural microbial flora of humans (Lister *et al.* 2009b, Mena and Gerba 2009, Morrison and Wenzel 1984). Despite its low frequency of colonization on the skin (0 – 2%), nasal mucosa (0 – 3.3%) and fecal samples (2.6 – 24%) of healthy individuals, *P. aeruginosa* infections are abundant and widespread in clinical settings (Morrison and Wenzel 1984), and is one of the three pathogens classified as a “critical threat” in the WHO list of antibiotic-resistant “priority pathogens” (Tacconelli and Magrini 2017). The bacterium is estimated to account for 10 – 20% of all hospital acquired infections, with immune-compromised individuals having a higher risk of infection (Bodey *et al.* 1983, Morrison and Wenzel 1984). *P. aeruginosa* is known to cause endocarditis, nosocomial pneumonia (14 – 16%), urinary tract infections (7 – 11%) and is a major cause of septicaemia (2 – 6%) (Gaynes and Edwards 2005, Kollef *et al.* 2005, Lister *et al.* 2009a, Richards *et al.* 1999). Through contaminated reservoirs, such as swimming pools and hot tubs as well as contact lens solutions, *P. aeruginosa* can also cause ear infections and keratitis (~13%) (Mena and Gerba 2009, Richards *et al.* 1999). Additionally, *P. aeruginosa* is commonly isolated from burn wound patients (~56%) and patients with cystic fibrosis (>60%), with its presence correlating to an increase mortality and morbidity in both cases (Bethesda 2007, Estahbanati *et al.* 2002).

Figure 1.1 – Biofilm associated infections caused by *P. aeruginosa*. *P. aeruginosa* plays a prominent role in cystic fibrosis pneumonia, where it forms biofilm-like microcolonies in the lungs of CF patients. *P. aeruginosa* is also commonly present in chronic wounds, resulting in delayed healing and increased inflammation. After *E. coli*, *P. aeruginosa* and *S. aureus* are the major contributors of chronic prostatitis. *P. aeruginosa* also plays a dominant role in medical device related infections, specifically infections on catheters, and implants of breast, penile and cochlear.

1.2 Overview of biofilms

Biofilms are surface associated bacterial cells encased in an extracellular polymeric matrix (EPS). Biofilms are inherently more resistant to antibiotic therapies (Section 1.5) and account for a large proportion of nosocomial implant-associated infections, chronic wound infections and chronic infections in cystic fibrosis patients (Bryers 2008, Parsek and Singh 2003). Biofilms in the environment could also act as reservoirs and possible sources of infections (Hall-Stoodley *et al.* 2004, Hall-Stoodley and Stoodley 2005).

Biofilm development, using *P. aeruginosa* as a model organism, generally includes three main stages, attachment, growth and maturation and dispersal (Figure 1.2), and may be mediated by signalling molecules in a cell-cell communication process known as quorum sensing (Li and Tian 2012) (Section

1.4.2). The processes leading to biofilm attachment and maturation are complex and have been extensively reviewed (Bryers 2008, Hall-Stoodley *et al.* 2004, Hall-Stoodley and Stoodley 2005, Parsek and Singh 2003). This chapter focuses on the dispersal stage of biofilm development.

Figure 1.2 – Stages of biofilm development with *P. aeruginosa* as the model organism. Biofilm development generally consists of five main stages – reversible (1) and irreversible (2) attachment to a surface with production of EPS, growth (3) and development of microcolonies (4) and dispersal of biofilm (5). The bottom panels show microscopic images of *P. aeruginosa* biofilms formed under continuous flow conditions on glass surfaces at various stages of biofilm development, with each image corresponding to a stage of development. This figure is reproduced with permission from Stoodley *et al.*, 2002.

1.3 General mechanisms of dispersal and phenotypes related to dispersed populations

1.3.1 Dispersal of biofilms – a passive or active process

Dispersal represents a distinct stage of biofilm development in which cells detach from the biofilm and transit from a sessile to a free-living, planktonic mode of life. This process is thought to help bacteria colonize new environmental niches and plays a role in the transmission of bacteria from the environment to host (Kaplan 2010).

Figure 1.3 – Biofilm dispersal processes. Erosion occurs when single or small clusters of cells dissociate from the biofilm while sloughing occurs when large proportions of cells detach from the biofilm. On the other hand, seeding dispersal occurs during the coordinated release of motile or non-aggregative cells from within mature microcolonies. Orange cells represent bacteria cells on the surface or exterior of the biofilm while green cells represent bacteria within microcolonies.

Dispersal occurs through a combination of passive and active process, with the former caused by external factors such as predation or physical factors such as fluid shear forces and abrasion (Choi and Morgenroth 2003, Lawrence *et al.* 2002, Stoodley *et al.* 2001, Wilson *et al.* 2004). Active dispersal, on the other hand, is mediated by the bacteria or biofilm and is typically associated with changes in gene expression or protein expression. Dispersal can be further classified into erosion, sloughing and seeding dispersal (Kaplan 2010) (Figure 1.3). During erosion, small clusters of cells are consistently dissociated from the biofilm surface. Sloughing occurs when large proportions of the biofilm are detached from the biofilm (Stoodley *et al.* 2001, Wilson *et al.* 2004). Both erosion and sloughing may be passively mediated. Lastly, seeding dispersal or central hollowing, occurs when a sudden large portion of highly motile or non-aggregative bacteria cells within matured microcolonies ‘exit’, leaving behind surrounding cells and a hollowed centre (Boles *et al.* 2005, Ma *et al.* 2009, Rice *et al.* 2008, Webb *et al.* 2003). Seeding dispersal has been observed in a variety of species, including but not limited to, *Actinobacillus actinomycetemcomitans*

(Kaplan *et al.* 2003), *Serratia marcescens* (Koh *et al.* 2007), *Staphylococcus aureus* (Yarwood *et al.* 2004), *P. aeruginosa* (Ma *et al.* 2009, Rice *et al.* 2008), *Pseudomonas putida* (Tolker-Nielsen *et al.* 2000) *Chromobacterium violacium*, *Caulobacter crescentus* and *Marinomonas mediterranea* (Mai-Prochnow *et al.* 2008). While the mechanism of central hollowing is not fully understood, it is thought to involve lysis of sub-populations of cells within the microcolony. Seeding dispersal has been associated with the generation of super-infective phages in *P. aeruginosa*, a buildup of reactive oxygen and nitrogen species (ROS and RNI) and the formation of variants with different phenotypes and colony morphology (Koh *et al.* 2007, Mai-Prochnow *et al.* 2008, Rice *et al.* 2008, Webb *et al.* 2003)

Active dispersal is generally regulated by cyclic-di-GMP (cdGMP) or quorum sensing (QS) through regulation of EPS degradation, activation of motility-related genes and upregulation of surfactant production. These mechanisms will be further discussed in sections 1.3.3. – 1.3.6.

1.3.2 Cyclic-di-GMP regulation of biofilm dispersal

Dispersal may be triggered by a variety of environmental cues, such as changes in nutrient levels (Huynh *et al.* 2012, Sauer *et al.* 2004), oxygen levels (An *et al.* 2010), iron availability (Banin *et al.* 2006, Lin *et al.* 2012, Nguyen *et al.* 2015, Rowe *et al.* 2010b) or the exogenous addition of nitric oxide (NO) (Barraud *et al.* 2006, Barraud *et al.* 2009b). Active dispersal may also be mediated by signal molecules such as acyl homoserine lactones (AHLs) (Dong *et al.* 2008, Hentzer *et al.* 2002, Purevdorj-Gage *et al.* 2005, Rice *et al.* 2005), fatty acid signals (Davies and Marques 2009, Dow *et al.* 2003) or peptide signals, i.e. autoinducing peptides (AIP) (Boles and Horswill 2008). These dispersal signals are sensed by receptors and translated to changes in the intracellular cyclic-di-GMP (cdGMP) concentration, which is known to play a role in transition of the bacteria between

the planktonic and biofilm modes of growth. Several papers have described in detail the role of cdGMP in regulating biofilm dispersal and formation. Although the effects of cdGMP may vary due to spatial and temporal regulation of DGCs and PDEs, in general, a higher cdGMP level is correlated with biofilm formation, while a low cdGMP level is linked to biofilm dispersal, motility and planktonic growth (Kulesekara *et al.* 2006, Römling *et al.* 2013, Valentini and Filloux 2016). This correlation has been shown in various bacterial species, including *P. aeruginosa*, *Escherichia coli* and *Salmonella enterica* serovar *Typhimurium* (Jenal and Malone 2006, Simm *et al.* 2004).

Figure 1.4 – Regulation of intracellular cdGMP levels and downstream effects. Synthesis of cdGMP is modulated by DGCs via a GGDEF domain while hydrolysis of cdGMP is modulated by PDEs containing an EAL or HD-GYP domain. DGC activity is promoted in the presence of GTP and inhibited by cdGMP, and may be further modulated by the presence of an I-site containing a RXXD motif. Intracellular cdGMP in turn regulates the downstream proteins, such as PleD, Alg44, LapD and FleQ, via binding to a DGC I-site, pilZ domain fold, degenerate EAL domain or an ATP binding site, respectively. By promoting the production of EPS components such as Pel, Psl and alginate, or preventing cleavage of adhesin proteins, cdGMP promotes the formation, or dispersal of biofilms.

CdGMP levels are regulated by both diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) (Römling *et al.* 2013). DGCs contain a conserved

GGDEF domain with a GG(D/E)F motif and synthesize cdGMP from two molecules of GTP while PDEs have conserved EAL or HD-GYP domains and break down cdGMP to pGpG and two molecules of GMP (Figure 1.4) (Römling *et al.* 2013, Valentini and Filloux 2016). Most bacteria encode multiple copies of DGCs and PDEs. For example, *P. aeruginosa* encodes 18 GGDEF, 5 EAL and 16 GGDEF/EAL containing proteins (Kulesekara *et al.* 2006, Valentini and Filloux 2016). These DGCs and PDEs may also contain additional domains involved in signal sensing or signal transmission, such as PAS, GAF or HAMP domains or hydrophobic regions involved in membrane localization (Valentini and Filloux 2016). Together, this suggests that there may be redundancy in the function of the proteins, that the proteins can modulate their activities in response to specific signals and that the protein or cdGMP may be temporally or spatially regulated (Christen *et al.* 2010).

Additional modulation of the DGC or PDE activity of an enzyme may also occur in proteins containing both DGC and PDE domains. In these proteins, called di-domain proteins, either or both DGC and PDE domains can be catalytically active, with the other serving a regulatory role (Christen *et al.* 2006, Jenal and Malone 2006). This is supported by the presence of a RXXD motif present 5 amino acids upstream of the GGDEF motif, which functions as an inhibitory site (I-site) for the allosteric control of cdGMP signaling (Christen *et al.* 2006). For example, evidence of allosteric control by the GGDEF domain has been demonstrated for several PDEs, including *P. aeruginosa* RbdA (An *et al.* 2010) and FimX (Kazmierczak *et al.* 2006) and *C. crescentus* CC3396 (Christen *et al.* 2005), in which the catalytically inactive GGDEF domain promotes cdGMP degradation in the presence of GTP. Furthermore, the *P. aeruginosa* protein MucR, which contains both functional GGDEF and EAL domains, acts as a positive regulator of alginate synthesis via its DGC activity under planktonic conditions while playing a role in dispersal via its PDE activity during biofilm growth (Li *et al.* 2013b). Eventually, cdGMP binds to, and allosterically regulates receptors, such as PleD, Arg44, LapD and FleQ, leading to specific cellular responses. These

responses may include PleD mediated production of EPS polysaccharide *pel* (Hickman and Harwood 2008, Lee *et al.* 2007, Li *et al.* 2012, Whitney *et al.* 2012), Alg44 mediated alginate synthesis (Merighi *et al.* 2007, Oglesby *et al.* 2008b), LapD mediated enzymatic degradation of the adhesin LapA via modulation of LapG (Gjermansen *et al.* 2010, Navarro *et al.* 2011, Newell *et al.* 2009, Newell *et al.* 2011, Rybtke *et al.* 2015) or FleQ regulation of motility-related genes (Amikam and Galperin 2006, Hickman and Harwood 2008).

1.3.3 Mechanisms of dispersal – Degradation of extracellular polymeric substances

The production of extracellular enzymes, such as proteases, glycosidases and nucleases, for the degradation of components of the biofilm extracellular polymeric substance (EPS) or biotic biofilm growth surfaces (Baty *et al.* 2000, Nijland *et al.* 2010, Pecharki *et al.* 2008), or degradation of points of attachment of the bacteria to the EPS (Finkelstein *et al.* 1992, Newell *et al.* 2011), is a common strategy utilized by diverse groups of bacteria for biofilm dispersal. The degradation of the matrix or points of attachment enable bacteria, that are otherwise encased within or adherent to the biofilm matrix, to be detached and released from the biofilm.

The role of cdGMP in mediating dispersal via such mechanisms has been reported for *P. putida* and *P. fluorescens* via cdGMP modulation of LapD activity (Gjermansen *et al.* 2010, Newell *et al.* 2009). This pathway is regulated by LapD, a protein that binds the periplasmic protease LapG, and the surface adhesin LapA, which mediates cell attachment to the biofilm. Under high cdGMP conditions, the secondary messenger is associated with LapD in a conformation suitable for binding LapG, that prevents LapG mediated degradation of LapA and hence promotes biofilm formation (Gjermansen *et al.* 2010, Navarro *et al.* 2011, Newell *et al.* 2009). Under phosphate limited conditions, expression of the PDEs RapA of *P. fluorescens*, or BifA of *P. putida*, results in a decrease in intracellular

cdGMP levels (Lopez-Sanchez *et al.* 2013, Monds *et al.* 2007). This leads to a conformational change in LapD and the release of LapG, which is then free to cleave LapA, promoting dissociation of the bacteria from the biofilm. Homologs of *lapD* and *lapG* are also present within the *P. aeruginosa* genome and have been found to play a role in dispersal via LapG cleavage of the non-homologous (LapA) adhesin CdrA (Rybtke *et al.* 2015).

In *P. aeruginosa*, expression of alginate lyase had also been found to increase biofilm detachment by breaking down alginate, an important component of the EPS in mucoid strains of *P. aeruginosa* (Boyd and Chakrabarty 1994). The exogenous addition of alginate lyase was found to increase the detachment of cells when administered at the time of inoculation and when administered to a pre-formed biofilm (Boyd and Chakrabarty 1994). In addition, the use of alginate lyase in combination with antibiotics, could enhance the eradication of *P. aeruginosa* in biofilms developed *in vitro* (Alkawash *et al.* 2006) or in rabbit endocarditis models (Bayer *et al.* 1992).

1.3.4 Mechanisms of dispersal – Activation of motility-related genes during dispersal

Dispersal induced through carbon or glucose starvation in *P. aeruginosa* biofilms has been found to coincide with an upregulation of motility genes (Huynh *et al.* 2012, Sauer *et al.* 2004). In *E. coli* biofilms, induction of *csrA*, a positive regulator of flagellar biosynthesis, was also found to lead to biofilm dispersal (Jackson *et al.* 2002). In general, increased flagellar motility is thought to facilitate cells in exiting from the central void during seeding dispersal (Sauer *et al.* 2002). However, upregulation of motility genes is not essential for dispersal (Morgan *et al.* 2006). In addition, non-motile bacteria, such as *S. aureus* and *A. actinomycetemcomitans*, can also undergo seeding dispersal (Kaplan *et al.* 2003, Yarwood *et al.* 2004). The former of which likely depends on an active *agr*

system and corresponding detergent-like peptides, protease and nuclease activity for release from the biofilm (Archer *et al.* 2011, Boles and Horswill 2008).

1.3.5 Mechanisms of dispersal – Surfactant mediated dispersal

The production of surfactants during dispersal had also been reported in *P. aeruginosa*, which is known to produce glycolipids known as rhamnolipids (Abdel-Mawgoud *et al.* 2010). Rhamnolipids contain one or two rhamnose glycosyl head group linked to 3-(hydroxyalkanoyloxy) alkanolic acid (HAA) fatty acid tails. Together, the head group and fatty acid tails give rise to the amphipathic properties of rhamnolipids, enabling them to act as extracellular surfactants (Abdel-Mawgoud *et al.* 2010). Dispersal by surfactants may be actively mediated via quorum sensing (QS) regulation of surfactant production, or passively through disruption of interactions between cells and surfaces.

Rhamnolipids, as well as the associated biosynthetic genes including *rhlA*, have been shown to play a role in biofilm formation, and the formation of distinct biofilm architecture in *P. aeruginosa*. This is supported by the observation that *rhlA* expression was significantly elevated in the stalk region of a mature biofilm microcolony (Boles *et al.* 2005, Davey *et al.* 2003, Lequette and Greenberg 2005, Pamp and Tolker-Nielsen 2007). In addition to their roles in biofilm formation, rhamnolipids have also been implicated in dispersal processes, as inactivation of genes involved in rhamnolipid synthesis, such as *rhlAB*, have been found to inhibit cell hollowing and detachment associated with seeding dispersal (Boles *et al.* 2005). The exogenous addition of rhamnolipids could also disperse *Bordetella bronchiseptica* (Irie *et al.* 2005) and *S. Typhimurium* biofilms (Mireles *et al.* 2001). Furthermore, rhamnolipids, together with the acylated homoserine lactone, 3oxoC12HSL, were found to mediate dispersal of *E. coli* biofilms exposed to *P. aeruginosa* supernatant, suggesting their roles in interspecies communication (Bhattacharjee *et al.* 2016).

1.3.6 Dispersal phenotypes

While dispersed cells have generally been thought to be similar to their planktonic counterparts (Sauer *et al.* 2002), a recent study by Chua *et al.* (2014) showed that dispersed cells are physiologically distinct from both planktonic and biofilm cells. In that study, dispersal was induced chemically using the NO donor compound, SNP, or enzymatically with a plasmid encoded PDE. Through transcriptomic based studies, several genes were shown to be differentially regulated in dispersed cells. *RsmY* and *rsmZ*, which are small regulatory RNAs that function antagonistically to c-di-GMP, and the pyoverdine related gene, *pvdA*, involved in iron uptake, were found to be down-regulated in dispersed cells. The downregulation of pyoverdine expression was also observed in other dispersal-related studies (Barraud *et al.* 2006, Barraud *et al.* 2009b). On the other hand, secretion and virulence genes, such as the type II secretion system (T2SS) genes *secB* and *secF*, were induced. Dispersed cells were more virulent to macrophages as compared to planktonic cells due to upregulation of the type III secretion system via repression of *rsmY*. In addition, dispersed cells were also more virulent to *Caenorhabditis elegans*, possibly due to upregulation of the T2SS. Increased virulence of dispersed cells were similarly observed in *Candida albicans*, in which dispersed cells displayed increased adherence to plastic surfaces and enhanced adhesion and damage to endothelial cells (Uppuluri *et al.* 2010). Lastly, it was shown that dispersed cells were sensitive to iron stress and the co-administration of an iron chelator with the antibiotic tobramycin, helped in the eradication of the bacteria, indicating that such combination therapy is an effective option against the highly virulent dispersal cells.

1.4 NO induced dispersal in *P. aeruginosa*

NO induced biofilm dispersal was first reported in *P. aeruginosa* in a study by Barraud *et al.* (2006). Subsequently, NO induced dispersal has been found to be

conserved in several other bacterial species, including but not limited to, *E. coli*, *Vibrio cholerae*, *S. aureus*, *Bacillus subtilis* and *Bacillus licheniformis* (Barraud *et al.* 2009b). While exposure to low NO concentrations may promote biofilm dispersal, NO is also known to exert a biphasic effect, leading to different outcomes, such as promoting biofilm formation, at higher concentrations (Barraud *et al.* 2006). Depending on the bacterial species and conditions, low concentrations of NO may also promote biofilm formation (Jardeleza *et al.* 2011). Therefore, understanding the pathways leading to NO induced dispersal requires understanding of NO sensing, regulation, production and elimination, which will be elaborated on in the following sections.

1.4.1 Response to NO is concentration and oxygen dependent

Several studies have reported conflicting results regarding the effects of NO on biofilm formation or dispersal. Indeed, the exogenous addition of NO could either promote biofilm formation (Barraud *et al.* 2006, Jardeleza *et al.* 2011, Plate and Marletta 2012b, Schmidt *et al.* 2004), or biofilm dispersal (Barnes *et al.* 2015, Barraud *et al.* 2006, Barraud *et al.* 2009b, Jardeleza *et al.* 2011, Li *et al.* 2013a, Roy *et al.* 2012, Schmidt *et al.* 2004). This may be due to the different NO concentrations used and oxygenation conditions (Arora *et al.* 2015), as high concentrations of NO may induce stress responses that promote biofilm formation while at low non-lethal concentrations, NO acts as a signaling molecule (Barraud *et al.* 2006, Jardeleza *et al.* 2011, Schmidt *et al.* 2004). Furthermore, oxygen levels influence the NO response pathways (Figure 1.5).

Under anaerobic conditions, in the presence of nitrate, *P. aeruginosa* can respire via denitrification, the dominant pathway for NO removal in anaerobic growth environments (Knowles 1982, Ye *et al.* 1994). During denitrification, NO is produced as a by-product by a nitrite reductase (NIR). NO is then converted to nitrous oxide by NO reductase (NOR) before being reduced to nitrogen gas by nitrous oxide reductase (NOS). Genes involved in denitrification also play a role

in biofilm formation and architecture under anaerobic conditions (Yoon *et al.* 2011), and will be elaborated on in subsequent sections (1.4.2).

Figure 1.5 – Effect of concentration of NO and presence of oxygen on NO response in *P. aeruginosa*. Depending on oxygen or NO levels, *P. aeruginosa* may induce different NO response pathways leading to NO detoxification via flavohemoglobin or denitrification enzymes, biofilm formation (at high NO concentration), biofilm dispersal (at low NO concentration), or stress response and adaptive mutations (at high ROS and/or RNI concentrations).

Under aerobic conditions, however, another pathway, involving the *fhp* gene, is required for NO detoxification (Arai *et al.* 2005). The *fhp* gene codes for flavohemoglobin, a conserved protein consisting of a bound heme and the redox cofactor, flavin adenine dinucleotide (FAD). The heme group of flavohemoglobin is thought to bind oxygen as a Fe^{3+} -O₂ intermediate that is poised to react rapidly with NO to form nitrate, hence detoxifying NO (Gardner *et al.* 1998). Expression of flavohemoglobin was found to be independent of denitrification, as *fhp* expression and NO detoxification were active in mutants of the dissimilatory nitrate respiration regulator (DNR), a master regulator of denitrification (Arai *et al.* 2005). Similarly, denitrification was independent of *fhp* regulation, where mutations in *fhpR* and *rpoN*, regulators of the *fhp* promoter,

did not affect promoter activities of DNR-dependent *nirS*, *norC* and *nosR* (Arai *et al.* 2005).

Oxygen levels influence the pathway of NO detoxification. In the presence of oxygen, reactive oxygen species (ROS) may also be formed. These reactive oxygen species can react with NO to give rise to highly reactive nitrogen species (RNI). In *P. aeruginosa*, peroxynitrite (ONOO-) has been detected in biofilms (Barraud *et al.* 2006). ONOO- is a reactive molecule that can induce DNA damage and cell death and is formed when a ROS, superoxide, reacts with NO. The formation of ONOO- is likely due to oxygen and NO gradients present within microcolonies of mature biofilms (Barraud *et al.* 2006, Chen *et al.* 2006). Like NO, low levels of ONOO- could induce dispersal without causing cell death (Barraud *et al.* 2006). However, at high concentrations, ROS may play a role in promoting mutations in *P. aeruginosa*, which may have implications in long-term colonization by and persistence of *P. aeruginosa* in the cystic fibrosis patients (Ciofu *et al.* 2005).

1.4.2 Regulation of biofilm development by genes of the denitrification pathway

1.4.2.1 NO and *nirS* are important for robust biofilm formation under anaerobic conditions and biofilm dispersal under aerobic conditions

Under anaerobic conditions, *P. aeruginosa* is known to form robust biofilms (Yoon *et al.* 2011, Yoon *et al.* 2002). The formation of biofilms was shown to be dependent on *lasR* and *rhlR*, transcriptional activators of the QS systems, as deletions of either or both genes were found to result in reduced biofilm formation under anaerobic conditions as compared to the wildtype strain. In contrast, these mutants were able to form thicker biofilms than the wildtype strains under aerobic conditions (Yoon *et al.* 2002). Further studies indicated that, in addition to forming less robust biofilms, cells within biofilms of *rhlR* and *lasRrhlR* mutants showed reduced viability (Yoon *et al.* 2002). Cell death was

likely caused by the accumulation of NO within the biofilms, as supplementation of carboxy-PTIO, a scavenger of NO, and double mutants of *rhlR* and NIR, which are unable to produce NO, were protected against cell death within the biofilms (Yoon *et al.* 2002). It was hence hypothesized that *rhlR* may regulate expression of the denitrification enzymes. Both NAR and NIR activities were increased in *rhlR* mutants and *rhlRrpoN* mutants as compared to the parental strain, with increases larger in the single mutant as compared to the double mutant. RpoN is a sigma factor that regulates and promotes *nirS* expression (Härtig and Zumft 1998) and detection of NIR activity in the double mutant indicated that *rhlR* is able to regulate *nirS* activity independent of RpoN (Yoon *et al.* 2002). In addition to regulation by *rhlR* and *lasR*, robust biofilm formation of *P. aeruginosa* under anaerobic conditions can also be directly linked to NO. NO accumulation was found to play a role in the formation of elongated cells under anaerobic conditions (Yoon *et al.* 2011). Elongated cells in turn facilitated the formation of cohesive clumps, which promoted biofilm formation. Cell elongation is thought to be specifically NIR-mediated, as deletion of *nirS*, but not *narG*, resulted in the abolishment of the elongation phenotype and no biofilm formation under denitrification conditions (Yoon *et al.* 2011).

In addition to its role in biofilm formation under anaerobic conditions, *nirS* is also important for dispersal in response to low doses of NO under aerobic conditions (Barraud *et al.* 2006). As with biofilm formation under anaerobic conditions, deletion of this gene abolished biofilm dispersal, even in the presence of the exogenously added NO donor SNP (Barraud *et al.* 2006). In contrast, a NOR mutant, which is defective in the removal of NO, displayed a hyper-dispersal phenotype (Barraud *et al.* 2006)

1.4.2.2 Denitrification is regulated by the ANR/DNR and QS systems

Quorum sensing (QS) is a cell-cell communication system through which bacteria coordinate population behaviors. *P. aeruginosa* is known to have four interconnected hierarchical QS systems, the well-studied *las* (Gambello and Iglewski 1991, Nealson *et al.* 1970, Stewart and Williams 1992) and *rhl* QS system (Pearson *et al.* 1995), the relatively newly discovered *Pseudomonas* quinolone signaling, *pqs* (Pesci *et al.* 1999) and *iqs* systems (Lee *et al.* 2013). Together, these four systems coordinate expression of virulence factors, such as iron acquisition genes, e.g. pyocyanin (Jensen *et al.* 2006, Lau *et al.* 2004), enzyme secretion (Gambello and Iglewski 1991, Wolz *et al.* 1994) and rhamnolipid production (Jensen *et al.* 2006). QS has also been implicated in several stages of biofilm development. In *P. aeruginosa*, mutants of QS systems, such as mutants of the LasI/LasR system, develop biofilms that are more sensitive to stress, likely due to the absence of *las* mediated expression of the biofilm matrix polysaccharide Pel (Colvin *et al.* 2011, Colvin *et al.* 2012, Sakuragi and Kolter 2007). The QS system also plays a role in biofilm dispersal via regulation of rhamnolipid production (Section 1.3.5). Likewise, QS has also been found to regulate nitrate respiration and plays a role in anaerobic biofilm formation (Section 1.4.2.1) Although the link between NO induced dispersal and QS is not well established, studies have described the regulation of denitrification by QS, and may shed light on how the processes of QS, denitrification and biofilm development intertwine with each other (Figure 1.6).

Denitrification is regulated by two transcription regulators, the oxygen transcriptional regulator ANR (anaerobic regulator of arginine deiminase and nitrate reductase) and the N-oxide transcriptional regulator DNR (dissimilatory nitrate respiration regulator). Under high oxygen conditions, oxygen inhibits the activity of ANR, which positively regulates itself, DNR and the two-component regulatory system NarXL (Schreiber *et al.* 2007). Together, ANR, DNR, and NarXL play a role in inducing expression from the *narK1* promoter of the

narK1K2GHJI operon that encodes the nitrate and nitrite transporters and NAR, which are required for denitrification (Schreiber *et al.* 2007). DNR, together with a functional NarXL system, is also required for transcription of *nirQ*, *nirS* and *norCB*, which encode NIR or NOR functions (Schreiber *et al.* 2007).

The regulation of denitrification by QS had been shown to be dependent on the *las* and *rhl* systems, and loss of function mutations within these systems increased denitrification (Hammond *et al.* 2015, Toyofuku *et al.* 2007, Yoon *et al.* 2002) via increased ANR activity (Hammond *et al.* 2015), and may lead to metabolic suicide through overproduction of NO (Yoon *et al.* 2002). The addition of 3OC12HSL, to $\Delta lasI$ strains that were unable to produce the signal molecule, also reduced ANR activity (Hammond *et al.* 2015). Similarly, ANR had been shown to regulate the expression of pathways related to QS. Together with LasR and RhlR, ANR has been shown to regulate the *hcnABC* operon involved in the production of hydrogen cyanide (Pessi and Haas 2000). Furthermore, in a *las* defective strain, ANR plays a role in the regulation of production of 4-hydroxy-2-heptylquinoline (HHQ), a precursor to the signaling molecule PQS of the PQS QS system. This in turn affected expression of iron acquisition pathways and increased production of CupA fimbriae (Hammond *et al.* 2015). While ANR may induce the *pqs* system, the *pqs* system may similarly influence the denitrification pathway. For example, the addition PQS has been shown to repress denitrification and promote NO accumulation by increasing the activity of NIR while inhibiting NOR activity. The effects that PQS exert on the nitrate-respiratory chain were independent of changes in transcription, suggesting that post-transcriptional controls were involved. Further, iron supplementation restored denitrification to normal levels, suggesting that it was the iron-chelating property of PQS that affected the process (Toyofuku *et al.* 2008). PQS is not produced under anaerobic conditions because PqsH, which oxidizes the PQS precursor HHQ, to PQS, requires oxygen as a cofactor (Schertzer *et al.* 2010). In addition, PQS production was also regulated by aeration, with lower PQS

production under microaerobic, as compared to aerobic conditions (Toyofuku *et al.* 2008). Although denitrification takes place primarily in anaerobic environments, it can also take place under oxygenated conditions (Chen *et al.* 2003), such as in oxygen limited environments of the lungs of CF patients (Worlitzsch *et al.* 2002). Taken together, PQS likely regulates denitrification under anoxic conditions.

Figure 1.5 – Regulation of denitrification by and between quorum sensing systems, ANR/DNR/NarXL systems, and effects of NO accumulation on biofilm formation and dispersal. The denitrification pathway has been adapted in part from (Alvarez *et al.* 2014), with green boxes showing the nitrogen species of denitrification and pink boxes showing the enzymes involved at each stage of denitrification. Denitrification is modulated by the *las*, *rhl* and PQS QS systems via effects on NAR (nitrate reductase), NIR (nitrite reductase) or NOR (NO reductase), or influence on the ANR and DNR transcriptional regulators. The ANR/DNR regulators, together with NarXL regulate transcription of denitrification genes. Oxygen plays a role in the process by promoting synthesis of PQS from the precursor HHQ by the enzyme PqsH and inhibiting the activity of ANR. Dotted lines refer to pathways where the mechanism is unknown, while solid lines refer to pathways which have been established. Arrow head ($\rightarrow$) represents an induction, while lines with straight lines ($\dashv$) represent an inhibition of protein activity or gene expression.

1.4.3 NO and its links to cyclic-di-GMP signaling

In addition to regulation by QS and denitrification related genes, NO may also influence biofilm development, specifically biofilm dispersal, through cdGMP signaling. During NO induced dispersal, increased in PDE activity, together with an accompanying decrease in cdGMP levels had been observed (Barraud *et al.* 2009a, Li *et al.* 2013a). Increases in PDE activity have been observed in cell-free extracts upon NO addition, indicating that modulation of PDE activities may be post-transcriptionally regulated (Barraud *et al.* 2009a). In addition, increased transcription of PDEs involved in NO induced dispersal had also been reported (Li *et al.* 2013a), suggesting that PDE mediated decreases in cdGMP during NO induced dispersal is regulated on multiple levels.

Figure 1.7 – Structural domains of PDEs involved in NO induced dispersal in *P. aeruginosa*. Domains of four PDEs known to be involved in NO induced dispersal, RbdA, DipA, NbdA and MucR. All proteins contain the GGDEF and EAL domains. However, only MucR is a functional DGC. RbdA, DipA and NbdA only display PDE activity. RbdA and DipA contain a cytosolic PAS sensor domain, while NbdA and MucR have a seven transmembrane (TM) MHYT sensor domain. In addition, DipA contains a GAF domain, which shares a similar fold to the PAS domain and likely plays a role in regulation of protein activity. Numbers above each domain indicates position of amino acids making up the domain while numbers at the end refers to the length of the protein.

Several PDEs, such as RbdA, DipA (Roy *et al.* 2012), NbdA, and MucR (Li *et al.* 2013a) were shown to be involved in NO induced dispersal, as isogenic deletions of these genes abolished the dispersal phenotype. All four proteins contain GGDEF/EAL domains, but only MucR contains both DGC and PDE activity, the activity of which is dependent on the growth phase of the bacteria (Li *et al.* 2013a). DipA and RbdA are similar in that both PDEs contain a Per-Arnt-Sint (PAS) domain, while NbdA and MucR contain a seven transmembrane (7TM) MHYT domain, in addition to the GGDEF/EAL domains. Except for NbdA, which was found to specifically be involved in NO induced dispersal, all three other PDEs are involved in dispersal to a variety of environmental cues, including NO and glutamate (Li *et al.* 2013a, Roy *et al.* 2012). Both the PAS domains and MHYT domains are important sensors of environmental signals (Galperin *et al.* 2001, Taylor and Zhulin 1999), of which, the PAS domain has been implicated in the sensing of light, diatomic gases such as oxygen (Gong *et al.* 1998) and NO (Rao *et al.* 2011) as well as redox potential in different proteins (Bibikov *et al.* 1997, Hill *et al.* 1996, Macheroux *et al.* 1998, Rebbapragada *et al.* 1997). RbdA, in particular, had been hypothesized to act as an oxygen sensor, due to the homology and conservation of residues necessary for heme binding when compared to the PAS domain of FixL, a known oxygen binding heme protein (An *et al.* 2010). PAS domains may also function as protein-protein interaction sites, as for DipA and RbdA interactions with BdlA, described below (Basu Roy and Sauer 2014). The MHYT domain, on the other hand, has been proposed to play a role in oxygen, carbon monoxide or NO sensing (Galperin *et al.* 2001).

Central to the role of RbdA and DipA in NO induced dispersal, is the involvement of a chemotaxis regulator, BdlA (biofilm dispersal locus A). Mutations of *bdlA* reduced the extent of NO induced dispersal (Barraud *et al.* 2009a), and gave rise to highly adherent biofilms with increased cdGMP levels (Morgan *et al.* 2006). The BdlA protein consists of two PAS domains, suggesting that BdlA, via its PAS domains, may act as a link between environmental cues, intracellular

cdGMP and dispersal (Morgan *et al.* 2006). In support of this, the PASa domain of BdlA was found to bind heme, and while heme binding may not be essential for the role of BdlA in biofilm dispersal, complementation of deletion mutants with *bdlA* containing a truncated PASa domain could not restore the dispersive phenotype in a *bdlA* mutant (Petrova and Sauer 2012b). BdlA was subsequently found to undergo a complex post-transcriptional regulation involving phosphorylation and protein cleavage before it can actively mediate dispersal (Petrova and Sauer 2012a). During this process, the protease ClpP, mediates cleavage of BdlA at a site between the two PAS domains only in the presence of increased cdGMP levels and when Tyr-238 was phosphorylated by the chaperone protein ClpD (Petrova and Sauer 2012a). Both cleavage products were required for the activity of BdlA and dispersal of *P. aeruginosa* (Petrova and Sauer 2012a). BdlA cleavage is additionally mediated by the PDE GcbA, which can interact with BdlA and a catalytically inactive GcbA variant was unable to rescue mutants with defective dispersal responses (Petrova *et al.* 2015). GcbA production was suppressed during biofilm development, with higher levels detected by qRT-PCR in planktonic cells than in biofilm cells. Further, GcbA production was induced either upon biofilm dispersal or when cdGMP levels were artificially reduced via overexpression of the dispersal specific PDE, *dipA* (Petrova *et al.* 2015). This induction of GcbA activity was more pronounced with overexpression of *dipA* as compared to overexpression of another PDE, PA2133, suggesting that dispersal specificity may play a role in the modulation of *gcbA*/GcbA expression (Petrova *et al.* 2015). Lastly, GcbA likely functions upstream of BdlA, as complementation of the isogenic mutant with truncated domains of BdlA was able to restore the dispersal phenotype (Petrova *et al.* 2015).

In a separate study of glutamate-induced dispersal in *P. aeruginosa* biofilms, a model between signal sensing and cdGMP hydrolysis was proposed for BdlA, involving the PDEs, RbdA and DipA (Basu Roy and Sauer 2014) (Figure 1.8). In this model, glutamate was thought to activate the glutamate-responsive DGC, NicD, via dephosphorylation of the protein. Activation of NicD leads to an initial

increase in intracellular cdGMP levels, which in turn leads to an initial increase in cdGMP levels that then contribute to the proteolytic activation of BdlA. Activated BdlA interacts with DipA, and perhaps RbdA, leading to an overall reduction in cdGMP levels, a prerequisite for *P. aeruginosa* biofilm dispersal (Basu Roy and Sauer 2014). Like NbdA and MucR, NicD contains a 7TM sensing domain, suggesting that NbdA and MucR may also function as external sensors of environmental cues and mediate dispersal through a similar pathway.

Figure 1.8 – Proposed pathway of biofilm dispersal via BdlA and PDEs involved in NO induced dispersal in *P. aeruginosa*. In *P. aeruginosa*, dispersal via glutamate is hypothesized to proceed via sensing of the glutamate signal by a DGC, NicD, followed by phosphorylation of the protein. Increased cdGMP, together with phosphorylation of NicD, inactivates the DGC, GcbA, and subsequent post-translational activation of BdlA. DipA and RbdA then interacts with BdlA, and their PDE activity reduces intracellular levels of cdGMP, leading to dispersal. Alternatively, NO is proposed to interact with NbdA or MucR, resulting in increased transcription of *nbdA*. NbdA then reduces cdGMP levels via its PDE activity, promoting dispersal of biofilms. Dotted lines refer to pathways where the mechanism is unknown. The figure is adapted, with modifications from (Basu Roy and Sauer 2014)

1.5 Strategies for application of NO induced dispersal

1.5.1 Resistance of biofilms to treatment

Biofilms can be up to 1000 fold more resistant to immune defenses and antibiotic therapies (Høiby *et al.* 2010, Stewart and Costerton 2001). Resistance of biofilms towards these treatments may arise due to the presence of an EPS, which acts as a barrier for reduced diffusion (de Beer *et al.* 1997, Jefferson *et al.* 2005), decreased antibiotic penetration (Davenport *et al.* 2014) or function as a pool for enzymes like β -lactamases, which are involved in hydrolyzing β -lactam antibiotics (Dibdin *et al.* 1996, Nichols *et al.* 1989). The heterogeneity of biofilms also contributes to their resistance, as gradients of nutrients, oxygen and reactive species give rise to populations of cells that are starved or have decreased growth rates and metabolic activity (Walters *et al.* 2003), or expression of genes promoting biofilm formation (Chua *et al.* 2014, Jang *et al.* 2016, Oh and Jeon 2014). Such slow or non-growing cells are inherently more resistant to drugs such as cephalosporin, aminoglycosides, and fluoroquinolones, as these antibiotics affect processes such as cell wall, protein, or DNA synthesis that are more active in growing cells. Repression of genes involved in detoxification of oxidative species may also increase the frequency of mutations through the accumulation of reactive oxygen or nitrogen species, which in turn promote the selection of resistant strains within biofilms (Driffield *et al.* 2008, Rodriguez-Rojas *et al.* 2012). Consequently, due to such resistance mechanisms, biofilms may be difficult to eradicate, leading to chronic, recurrent infections within the host.

1.5.2 Strategies for biofilm control

Strategies for biofilm control generally aim to prevent biofilm formation or disperse established biofilms. Towards this end, QS inhibitors (QSI) may be used to disrupt cell-cell communication involved in biofilm formation and development. QS inhibitors, such as halogenated furanones derived from the

marine algae *Delisea pulchra*, have been shown to reduce the concentration of a QS responsive transcriptional regulator LuxR when expressed in *E. coli* (Manefield *et al.* 2002). Furanones also enhanced bacterial detachment of *P. aeruginosa* biofilms (Hentzer *et al.* 2002). Further studies of the effects of furanones on *Aeromonas hydrophila*, a bacterial strain found in reverse osmosis membranes, indicated that these compounds were effective against biofilm formation and could potentially be applied as a QSI in bacterial biofilm communities to prevent biofouling (Ponnusamy *et al.* 2010). In addition to furanones, 2-aminobenzimidazole derivatives have also been shown to interfere with *P. aeruginosa* *las* and *rhl* QS, inhibiting biofilm formation and promoting dispersal (Frei *et al.* 2012). Besides the use of QSI, enzymatic methods, such as treatment with proteases and alginate lyase, had also been employed to break down the EPS and encourage biofilm removal (Alkawash *et al.* 2006, Bayer *et al.* 1992, Boyd and Chakrabarty 1994, Lequette *et al.* 2010). The combination use of alginate lyase for this purpose, together with the antibiotic gentamycin, was also shown to promote eradication of biofilms (Alkawash *et al.* 2006). Protein engineering methods have also been developed to allow manipulation of intracellular cdiGMP levels to induce biofilm dispersal (Ma *et al.* 2011). Lastly, NO could be used for chemically inducing biofilm dispersal. To date, several systems have been developed and evaluated for the delivery of NO to biofilms (Section 1.5.6).

Figure 1.9 – Mechanisms of biofilm resistance to antibiotic therapies and strategies for biofilm control. Biofilms are inherently resistant to antibiotic treatments due to the presence of EPS, and heterogeneity within biofilms due to gradients that arise within biofilms. Strategies for biofilm control involve prevention of biofilm formation, usually using QS inhibitors, or induction of biofilm dispersal through enzymatic treatments, modulation, or via the use of NO as a signal for dispersal.

1.5.3 NO and host immune response

(In this paragraph, NOS refers to NO synthase, not nitrous oxide reductase of the denitrification pathway, which is also commonly abbreviated as NOS.)

This section briefly describes the levels of regulation of NO during immune response (Figure 1.10). NO regulation during immune and inflammatory responses is highly complex, and more in-depth reviews have been published (Bogdan 2001a, Bogdan 2001b, Bogdan 2015, Coleman 2001). Within a host, NO is involved in many immune or inflammatory processes through the activity of inducible NO synthase (iNOS) and constitutive NOS (cNOS), proteins that synthesize NO from L-arginine. These NO regulated processes may include immune cell differentiation and proliferation (Niedbala *et al.* 1999), cytokine production (Bauer *et al.* 1997, Bogdan 2001a, Marshall *et al.* 2000, Niedbala *et*

al. 1999) and facilitate leukocyte recruitment and adhesion (Kubes *et al.* 1991). NOS can be found in both immune cells such as dendritic, natural killer, mast cells and phagocytic cells as well as non-immune cells involved in immune response, such as endothelial, epithelial, vascular smooth muscle cells and fibroblasts, where they mediate the above-mentioned processes (Bogdan 2001b).

Figure 1.10– Involvement of NO and iNOS in immune responses. In addition to its role in bacterial biofilms. NO also acts as a signal in mammalian systems. In mammalian immune systems, NO is produced by iNOS or cNOS from L arginine, where it plays several roles, such as the induction of cytokine production or antimicrobial activity. Regulation of NO production is complex, as different cytokines promote or inhibit the activity of GTP cyclohydrolase, which synthesizes BH₄, a cofactor of NOS. Furthermore, iNOS activity is promoted in the presence of pathogenic materials and by the Nf-κβ, and inhibited by NLRP3. NLRP3 and Nf-κβ is in turn regulated by NO in a concentration dependent manner, where high levels of NO results in the nitrosylation and inhibition of NLRP3 and low levels of NO promote the activity of Nf-κβ. Dotted lines refer to pathways where the mechanism is unknown, while solid lines refer to established pathways. Arrow head (→) represents an induction, while lines with straight lines (⊥) represent an inhibition of protein activity.

During an infection, NO production in macrophages is induced in the presence of bacterial or viral proteins, such as the effector protein SopE2 of *Salmonella typhimurium* (Cherayil *et al.* 2000), or pathogen derived DNA and

lipopolysaccharide (LPS) (Gao *et al.* 1999, Shoda *et al.* 2001). NO synthesis is also indirectly regulated by cytokines like IFN- γ , TNF, IL-1 and IL-4, as the cytokines regulate the activity of guanosine triphosphate cyclohydrolases, enzymes involved in the synthesis of tetrahydrobiopterin, also known as BH₄, an allosteric regulator of NOS activity (Bogdan 2001b, Hattori *et al.* 1997). In addition to regulation via external stimuli or via cytokine signaling, iNOS activity may also be modulated via NO. At low NO concentrations, NO activates the pro-inflammatory transcription factor Nf- κ B, which upregulates the transcription of iNOS (Bogdan 2001a, Umansky *et al.* 1998). At high NO concentrations, however, NO exerts an inhibitory effect on iNOS production via S-nitrosylation of the inflammasome protein NLRP3 (Hernandez-Cuellar *et al.* 2012, Mao *et al.* 2013, Rayamajhi and Miao 2013). Together, these suggest the presence of multiple levels of regulation of NOS activity during immune response. Consequently, NO regulated processes may have different effects that are antiviral and antimicrobial, pro- or anti-inflammatory, and cytotoxic or cytoprotective (Bogdan 2001b). As such, depending on the pathogen species or strain, infection dose, entry route or phase of infection, iNOS activity may help alleviate or aggravate the infection or have no significant effects at all (Bogdan 2001b).

1.5.4 NO and pathology of cystic fibrosis

While iNOS activity may lead to different prognosis during an infection, in general, reduced iNOS expression and NO levels have been correlated to disease progression in cystic fibrosis patients (Balfour-Lynn *et al.* 1996, Grasemann *et al.* 1997, Michl *et al.* 2013, Moeller *et al.* 2006, Thomas *et al.* 2000). Measurements of exhaled NO levels may be affected by multiple factors such as the presence of a thick mucus that may retard diffusion of NO out of the airways (Balfour-Lynn *et al.* 1996), utilization of NO in denitrification processes by *P. aeruginosa* in an anaerobic or microaerobic environment (Balfour-Lynn *et al.*

1996, Meng *et al.* 1998, Warren *et al.* 1990), lowered levels of L-arginine, which is a precursor of NO synthesis (Grasemann *et al.* 2005) and the changes in activity of iNOS in CF patients (Meng *et al.* 1998). In children diagnosed with CF, iNOS mRNA expression was found to be significantly lower in CF patients regardless of the presence of infection, as compared to healthy children not diagnosed with CF (Moeller *et al.* 2006). Such low mRNA expression coincided with a decreased iNOS protein accumulation and suggested that low iNOS expression may affect local antimicrobial defenses and epithelial cell function (Moeller *et al.* 2006).

1.5.6 NO for control of biofilm infections

NO has been studied as a biofilm control agent using different delivery systems. The effects of NO treatment may be multi-pronged, preventing biofilm formation by reducing bacteria adhesion to surfaces (Charville *et al.* 2008), inducing biofilm dispersal at low concentrations (Barraud *et al.* 2006), having antimicrobial effects at high concentrations (De Groote and Fang 1995, Schairer *et al.* 2012) or modulating local immune responses (Bogdan 2001b). Appendix A provides a summary of NO donors and delivery systems evaluated for use in biofilm control.

1.5.6.1 NO gas

As a gas, NO could be administered directly to an accessible site of infection, such as in the case of a skin infection (Ghaffari *et al.* 2007, Miller *et al.* 2004) or administered via release from wound dressings (Sulemankhil *et al.* 2012) (~200 ppm NO were used in each study). Direct administration of gaseous NO to wounds or ulcers have been shown to reduce bacterial load at the site of infection, likely through antimicrobial activities of NO (Ghaffari *et al.* 2007, Miller *et al.* 2004, Sulemankhil *et al.* 2012), and promote wound healing (Miller *et al.* 2004) via NO and iNOS regulation of collagen production, cell proliferation and wound

contraction (Witte and Barbul 2002). In addition, gaseous NO can be inhaled directly for treatment of lung infections. In proof-of-concept clinical studies, low dose of NO gas (~5 ppm, equivalent to ~200 mM NO) were delivered to CF patients, together with intravenous tobramycin and ceftazidime, over 7 – 8 days, and was found to reduce *P. aeruginosa* biofilms and improve lung function when compared to placebo-treated control patients (Howlin *et al.* 2017). Exposure to higher NO doses of 10 or 40 ppm were also found to reduce infiltration of *P. aeruginosa* and help clear lung infections in rats (Jean *et al.* 2002, Webert *et al.* 2000). Similarly, higher doses of NO (160 ppm) significantly reduced *P. aeruginosa* bacterial load in rat pneumonia models (Miller *et al.* 2013). While administration of 160 ppm of gaseous NO treatment may be directly biocidal, antibacterial effects of 40 ppm of NO treatment occur indirectly by stimulating the host immune system (Miller *et al.* 2013).

1.5.6.2 NO donors

Besides direct application as a gas, NO could also be delivered by means of NO donors, which are compounds that can liberate NO under aqueous physiological conditions either spontaneously or following enzymatic activation (Miller and Megson 2007, Schonafinger 1999). Several classes of NO donors have been developed and extensively studied, such as diazeniumdiolates (NONOates), metal-NO complexes, and organic nitrates or nitrites, for their use as mainly vasodilators and atherosclerosis prevention agents (Miller and Megson 2007, Schonafinger 1999). The classes of NO donors have different mechanisms of NO release, NO release kinetics, pharmacological and chemical properties that affect compound stability, routes of administration, as well as the development of resistance against the NO donors. The availability of different classes of NO donors hence enables application of different NO donors for treatment of a range of pathological conditions (Miller and Megson 2007, Schonafinger 1999).

1.6.6.2.1 Metal-NO complexes – Sodium nitroprusside

Metal-NO complexes, such as sodium nitroprusside (SNP) has been shown to disperse single species *P. aeruginosa* biofilms grown under flow conditions at 500 nM (Barraud *et al.* 2006), with higher SNP concentrations used in other studies (Li *et al.* 2013a, Roy *et al.* 2012). The use of SNP to disperse biofilms has also been shown to potentiate the effects of antibiotics like tobramycin or ceftazidime in eradication of *P. aeruginosa* biofilms grown on abiotic surfaces and ex vivo sputum samples (Barraud *et al.* 2006, Howlin *et al.* 2011). In addition to the eradication of *P. aeruginosa* biofilms, SNP also effectively removed biofilms formed by other bacterial or fungal species, such as the gram negative bacteria *E. coli*, *V. cholerae*, *S. marcescens*; Gram-positive bacteria such as *B. licheniformis* and *S. epidermidis* and the yeast *C. albicans* (Barraud *et al.* 2009b). While SNP has been considered one of the most essential medicines by the World Health Organization and has been used extensively clinically (WHO 2015), the mechanism of NO release from SNP is not fully elucidated and requires either light or tissue activation (Miller and Megson 2007). NO release from SNP is also cyanogenic, and involved the release of cyanide ions from the SNP parent compound, with conversion of hemoglobin to cyanmethemoglobin (Hottinger *et al.* 2014).

1.6.6.2.2 Diazeniumdiolates (NONOates)

Unlike SNP, NO release from NONOates follow first-order kinetics dependent on temperature and pH (Keefer 2011, Morley and Keefer 1993), with the rate of decomposition dependent on the polyamine – R group (Hrabie *et al.* 1993, Keefer 2011). Due to the predictability of NO release from NONOates, they have been tested in various settings for their effectiveness in dispersing biofilms (Barnes *et al.* 2015, Barraud *et al.* 2009b, Marvasi *et al.* 2014). NONOates are also often used in studies involving NO due to their low or moderate toxicity, low probability of carcinogenicity, low cost and commercial availability (Marvasi *et al.* 2014).

NONOates have been studied for their application in membrane systems. In one such study, Proli NONOate treatment was found to reduced membrane surface coverage by bacterial found in industrial membrane bioreactor and reverse osmosis membranes, which delayed fouling by increasing the amount of time leading to maximum transmembrane pressure. The reduction was accompanied by a decrease in average biofilm thickness and quantity of EPS components, including polysaccharides and proteins, with no effects on membrane bacterial community (Barnes *et al.* 2015). MAHMA NONOate, or NOC-9, was similarly found to reduce both single and mixed species biofilms formed from isolates of industrial membrane reactors and reverse osmosis membranes (Barnes *et al.* 2013). In addition, MAHMA NONOate and a variety of NONOates, including diethylamine NONOate (DEA NONOate), diethylamine NONOate diethylammonium salt and spermine NONOate (S150, NOC-22) could also effectively disperse biofilms of food-borne pathogens like *Salmonella enterica* and pathogenic enterohemorrhagic *E. coli* O157:H7 on abiotic plastic surfaces, which are common surfaces of food preparation (Marvasi *et al.* 2014, Marvasi *et al.* 2016). Dispersal varied with the type of NONOates used, with differences in optimal conditions to which dispersal is induced (Marvasi *et al.* 2014). The NONOates also displayed synergistic effects when used in combination with sanitizers, and were found to promote dispersal of *Salmonella* biofilms by 20% and disperse week old *Salmonella* biofilms, which were not dispersed when MAHMA NONOate or the sanitizer was used alone (Marvasi *et al.* 2014)

1.6.6.2.3 Other NO donor compounds

Other NO donors have also been evaluated for their abilities to induce biofilm dispersal. This includes the S-nitrosothiol derived NO donors S-Nitrosoglutathione (GNSO) and S-Nitroso-N-acetyl-DL-penicillamine (SNAP), which effectively removed *S. marcescens* and *V. cholerae* biofilms (Barraud *et al.* 2009b). The extent of biofilm removal, however, was much lower than the extent induced with SNP, which was also evaluated in the same study (Barraud

et al. 2009b). SNAP was also evaluated as a dispersing agent at low temperatures, decreasing biomasses of *S. enterica*, *E. coli* and *Listeria innocua* by up to 30% when applied at 4°C (Marvasi *et al.* 2016).

Molsidomine, a sydnone imine, has also been evaluated as a biofilm dispersing agent. Molsidomine is an orally administered drug prescribed as a coronary vasodilator. NO release from molsidomine is dependent on the formation of an unstable compound, linsidomine, which is produced when molsidomine is metabolized in the liver. Interestingly, the compound effectively induced dispersal of *Salmonella* biofilms at both room temperature and at 4°C *in vitro*, suggesting that activation of the compound and its application can occur even at low temperatures (Marvasi *et al.* 2016). Additionally, molsidomine was effective at dispersing *Pectobacterium carotovorum* biofilms, especially when used in combination with a sanitizer, peracetic acid. Together, the study suggests that combination application of NO donors and sanitizers could improve sanitization of refrigerate environments (Marvasi *et al.* 2016).

1.5.6.3 Hybrid compounds

Hybrid compounds that combine a QS inhibitor, fimbrolide, linked to a NO donor, such as a nitrooxy groups or NONOates have been studied (Kutty *et al.* 2013). As briefly described in Section 1.4.2.2, QS plays important roles in biofilm formation and development. Combining a moiety that targets QS and a NO donor group that acts to inhibit biofilm formation or induce dispersal hence enable synergistic effects against targets. Such fimbrolide-NO donor hybrids displayed higher activities with regards to repressing of QS as compared to the parent fimbrolide. Depending on the NO donor group, fimbrolide-NO donor hybrids were also found to inhibit biofilm formation to a greater extent than the reference NO donor, SNP (Kutty *et al.* 2013). Taken together, the hybrid hence represents a dual-action antimicrobial agent that interferes with both QS and NO signalling for controlling bacterial virulence without inhibiting bacterial growth.

Another advantage of hybrid compounds is the pharmacokinetic characteristics of one component of the hybrid can be controlled by the linkage moiety. Such is in the case of cephalosporin-linked diazeniumdiolate hybrid prodrugs, in which a cephalosporin group is linked to a NONOate component (Barraud *et al.* 2012, Collins *et al.* 2017). On its own, the NONOate spontaneously decomposes in aqueous solutions under physiological conditions (section 1.5.6.2). However, linkage to the cephalosporin moiety generates a stable prodrug that is unable to spontaneously decompose to release NO in solution. Activation of the hybrid occurs when the prodrug is cleaved by a β -lactamase (Barraud *et al.* 2012, Collins *et al.* 2017), a bacterial specific enzyme that may account for resistance of biofilms to antibiotics (see section 1.5.1). NO release from cephalosporin-linked diazeniumdiolate compounds have been shown to mediate biofilm dispersal (Barraud *et al.* 2012) and increase cell susceptibility to other antimicrobial treatments (Barraud *et al.* 2009b, Collins *et al.* 2017). In all, these hybrid compounds enable targeted delivery and release of NO close to the site of biofilm infection, inducing dispersal or promoting eradication of biofilm by other antimicrobials, and reducing non-specific effects of NO in the host.

1.5.6.4 NO release from coatings or surfaces

In addition to direct application as a gas or release of NO in solution via NO donors, NO could also be released from coated surfaces. In a study evaluating the effects NO release from xerogel polymers coated with fibrinogen, a glycoprotein present in blood plasma that can mediate implant associated infections, NO flux on the fibrinogen coated polymer surface was found to correlate strongly to reduction of *S. aureus*, *S. epidermidis* and *E. coli* adhesion (Charville *et al.* 2008). While the reduction in *S. aureus* and *E. coli* adhesion was found to be dependent on the flux of NO from 0 – 30 pmol/cm/s, reduction of *S. epidermidis* adhesion was mainly observed upwards of 18 pmol/cm/s, suggesting that, under the conditions tested, NO release from fibrinogen-coated surfaces were more

effective against *S. aureus* and *E. coli* as compared to *S. epidermidis* (Charville *et al.* 2008).

NO may also be designed to be released from silica or gold-based nanoparticles at both high and low concentrations (Duong *et al.* 2014, Hetrick *et al.* 2008). NO release from nanoparticles have been shown to reduce *P. aeruginosa* biofilm surface coverage on polystyrene surfaces (Duong *et al.* 2014). In addition, controlled NO release from nanoparticles were also shown to be more effective at killing planktonic *P. aeruginosa* cells with less toxicity exhibited to mammalian cells (Hetrick *et al.* 2008). Versatility in the synthesis of scaffolds, or nanoparticles for NO release may further enhance their role as antibacterial or anti-biofilm agents.

1.6 Summary

Biofilms represent the predominant lifestyle of bacteria found in natural environments. Such biofilms are highly resistant to antibiotic treatments and strategies for biofilm control involve inhibition of biofilm formation and/or induction of biofilm dispersal (Barraud *et al.* 2015). With focus on dispersal, studies on biofilm have revealed strategies and signals that the bacteria use to coordinate biofilm dispersal, including disruption of the EPS, production of surfactants and response to NO. These mechanisms can be exploited for the control of biofilms, as enzymes such as alginate lyases or exogenous NO are used to induced dispersal (Alkawash *et al.* 2006, Barraud *et al.* 2015). Several studies have focused on the use different NO donors on biofilm dispersal, with focus on the use of NO gas, SNP or NONOates with short half-lives. While NO application is a viable option for the control of biofilms in both industrial and clinical settings, issues, such as the short half-lives of compounds, reactivity of NO with other chemical groups, toxicity of donor compounds, efficacy *in vivo* and secondary effects within mammalian systems remains to be addressed.

1.7 Objective and scope of study

Biofilm dispersal has been identified as one of the means of control of biofilm infections (Barraud *et al.* 2015). To this end, nitric oxide (NO) has been recognized for its application as a dispersing agent for biofilm control (Barraud *et al.* 2015). Because of the ubiquitous nature of NO and its role in pathological conditions such as hypertension and angina, a wide range of NO donor compounds have been developed for the treatment of such conditions (Hampl and Herget 2000, Marin and Rodriguez-Martinez 1997, Miller and Megson 2007). These classes of compounds have different chemical stability, NO kinetics, mechanism of NO release and duration of action that may be advantageous under different conditions (Miller and Megson 2007). While the applications of these NO donors in human disorders have been well-studied, many classes of NO donors have not been evaluated for their activity against biofilms. In addition, due to the reactivity of NO, there is a need to explore alternative methods of NO delivery that enable targeted delivery of NO to bacteria biofilms and reduce non-specific reactions. Lastly, it is of interest to understand how NO is sensed by *P. aeruginosa* and the pathways leading up to dispersal as further understanding of the mechanisms of NO induced dispersal could facilitate optimization of the use of NO treatment for biofilm control. The objectives and scope of this project are hence to:

1. Characterize the effects of a novel class of furoxan based NO donors on model *in vitro* *P. aeruginosa* biofilms;
2. Evaluate the effectiveness of cephalosporin-linked NO donor pro-drugs that enable targeted delivery of NO to bacteria biofilms using both *in vitro* plate models and *in vivo* animal models and;
3. Understand the mechanism of NO induced dispersal by evaluating the role of RbdA and DipA as sensors of NO through protein studies and assessing the roles of other proteins in NO induced dispersal.

2. Furoxan-derivative NO donors

The work presented in this chapter has been published and is reformatted here from: Poh WH, Barraud N, Fruttero R, Rice SA. Furoxan Nitric Oxide Donors Disperse *Pseudomonas aeruginosa* Biofilms, Accelerate Growth, and Repress Pyoverdine Production. ACS Chemical Biology 2017; accepted 20-06-17. doi: 10.1021/acscchembio.7b00256.

2.1 Introduction

Bacterial cells growing within biofilms can be up to a thousand times more resistant to anti-microbial agents than their planktonic counterparts, making their eradication very difficult (Høiby *et al.* 2010, Stewart and Costerton 2001). This enhanced tolerance to stress, immune defenses and antibiotics may partly explain the observation that biofilms are associated with up to 80% of all microbial infections and the majority of chronic and recurrent infections including pneumonia in cystic fibrosis patients, wound infections and medical implant-associated infections (Høiby *et al.* 2010).

The insensitivity of biofilms to conventional antimicrobial treatments appears to be multifactorial and involves: (i) a protective barrier of self-produced extracellular polymeric substances (EPS) that can inactivate or reduce diffusion of bactericidal compounds (Mulcahy *et al.* 2008), (ii) enhanced lateral transfer of genes including those from drug resistant and extremely drug resistant (XDR) strains (Roberts and Mullany 2010) and (iii) the presence of dormant-like persister cells (Lewis 2007). Given the central role played by biofilms in promoting antibiotic resistance and causing the failure of therapeutic treatments, there is an urgent need to develop alternative strategies specifically aimed at biofilm development processes. Towards this end, the dispersal phase of the biofilm life cycle has been targeted as a key stage that can be manipulated to control biofilms (Barraud *et al.* 2015). During dispersal, bacterial cells transit from a biofilm to a planktonic mode of growth, rendering them more susceptible

to various antibiotics (Barraud *et al.* 2006, Howlin *et al.* 2011). Biofilms disperse in response to several environmental cues, such as changes in carbon levels and iron levels (Gjermansen *et al.* 2005, Huynh *et al.* 2012, Rowe *et al.* 2010a). In addition, low nanomolar concentrations of the gaseous free radical nitric oxide (NO) were found to induce biofilm dispersal in *Pseudomonas aeruginosa*, an opportunistic pathogen and the model organism for biofilm studies (Barraud *et al.* 2006), as well as several other bacterial species (Barraud *et al.* 2015). Furthermore, exposure of pre-established biofilms to the NO donor compound sodium nitroprusside (SNP) increased the susceptibility of biofilm cells towards several antimicrobial agents, including the antibiotics tobramycin and ceftazidime in *P. aeruginosa* biofilms grown both on abiotic surfaces and in ex vivo sputum samples of CF patients (Barraud *et al.* 2006, Howlin *et al.* 2011). Recently, the use of NO gas delivered at low dose to CF patients, together with intravenous tobramycin and ceftazidime, reduced *P. aeruginosa* biofilms and improved lung function when compared to placebo-treated control patients in proof of concept clinical studies (Howlin *et al.* 2017, Katrina *et al.* 2014). These observations suggest that the use of NO, together with conventional antibiotics, represents a promising alternative for the treatment of chronic biofilm infections.

The delivery of NO for medical purposes, in addition to the gaseous form, is typically achieved by using soluble donors. These compounds spontaneously release NO when dissolved in aqueous solutions and the kinetics of release are a function of their donor chemistry. In this way, donors can be tuned to optimize the release of NO. Furoxans are heterocyclic compounds containing a 1,2,5-oxadiazole 2-oxide ring and two substituent groups at positions 3 and 4 of the furoxan ring (Figure 2.1) (Gasco *et al.* 2004). These substituents influence several properties of the furoxan compound, such as its solubility, the rate of NO production, and whether NO-release is thiol-activated or could occur spontaneously (Feelisch *et al.* 1992a, Gasco *et al.* 2004, Sorba *et al.* 1997). This flexibility allows for the design of furoxan compounds that generate varying

fluxes of NO when administered (Sorba *et al.* 1997). In general, furoxans have an advantageous pharmacological profile of a slow onset with an extended duration of action, as compared to other NO donors (Gasco *et al.* 2004, Schönafinger 1999). In turn, furoxans as such, or used in designing NO donor hybrid drugs, have been observed to be active against a variety of targets and have been assessed for use in cardiovascular diseases, neurological and inflammatory disorders (Boschi *et al.* 2006, Cena *et al.* 2003, Hugo and Williams 2005). More recently, a few furoxan derivatives were found to inhibit ABC transporters in MDR tumor cells (Chegaev *et al.* 2011, Fruttero *et al.* 2010)

Despite the many applications of furoxans, they have not been tested for their activity against biofilms. In this study, we evaluated the release of nitrogen oxide species (NO_x), namely NO and nitrite (NO_2^-), from three furoxans, LL4212, LL4216 and LL4254, and studied their effect on *P. aeruginosa* biofilm development, where they induced biofilm dispersal. LL4212 and LL4216 were, additionally found to affect bacterial growth under iron-limited conditions in an NO_x -independent manner, increasing the rates of glucose utilization and in turn leading to the earlier onset of glucose-starvation-induced dispersal of *P. aeruginosa* biofilms. Further, LL4216 was found to reduce expression of the siderophore pyoverdine in an NO_x -independent manner. Taken together, the study indicated that, depending on their backbone, furoxans may have secondary effects on bacterial growth and is an important consideration for their design and use as NO-releasing agents.

2.2 Materials and methods

2.2.1 Nitric oxide donors and scavengers

The furoxans LL4212 (3-((2-(dimethylamino)ethyl)oxy)-4-phenylfuroxan), LL4216 (3-((2-aminoethyl)thio)-4-phenylfuroxan) and LL4254 (4-(phenylsulfonyl)-3-((2-(dimethylamino)ethyl)thio)furoxan) were synthesized as previously reported by Sorba *et al.* (Sorba *et al.* 1997). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (carboxy-PTIO, cPTIO) was purchased from Sigma Aldrich (# C221).

Figure 2.1 – Structures of LL4254 (a), LL4212 (b) and LL4216 (c) used in this study. The compounds contain a 1,2,5-oxadiazole 2-oxide five-ring structure and two substituent groups at positions 3 and 4 of the furoxan ring.

2.2.2 Nitrite measurements by Griess reaction (total NO_x evaluation)

The total release of NO_x was evaluated as nitrite (NO₂⁻) by Griess reaction. Furoxan compounds were incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration in the absence or in the presence of 0.5 mM L-cysteine (L-cys) (5 times mol/mol excess). At regular intervals, the presence of nitrite in the reaction mixture was determined by the Griess assay: 1 mL of the reaction mixture was treated with 250 µL of the Griess reagent (4% w/v sulphanilamide, 0.2% w/v *N*-naphthylethylenediamine dihydrochloride, 1.47 M phosphoric acid). After 10 min at room temperature, the absorbance was measured at 540 nm. A

calibration curve was obtained using standard solutions of sodium nitrite at 10 μM to 80 μM . The yield in nitrite was expressed as percent NO_2^- (mol/mol, relative to the initial compound concentration) $\pm$ SEM.

2.2.3 NO measurements by DAN (2,3-diaminonaphthalene) method

NO release was quantified using a 2,3-diaminonaphthalene (DAN)-based chemical assay, which is based on the immediate reaction of NO with oxygen (O_2) to form dinitrogen trioxide (N_2O_3), which then reacts with non-fluorescent DAN to form the highly fluorescent 2,3-naphthotriazole (NAT) that can be quantified by RP-HPLC. Compounds were incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration with 0.2 mM DAN in the absence or presence of L-cys at 0.5 mM. At fixed time points, NAT in the reaction mixture was evaluated by HPLC analysis according to previously published protocol (Fang *et al.* 2009).

HPLC analyses were performed with a HP 1200 chromatograph system (Agilent Technologies) equipped with a quaternary pump (G1311A), a membrane degasser (G1322A), a multiple wavelength UV detector (G1365D) and a fluorescence detector (G1321A) integrated in the HP1200 system. Data analysis was performed using a HP ChemStation system (Agilent Technologies). The sample was eluted on a Zorbax Eclipse XDB-C18 column (150×4.6 mm, 5 μm ; Agilent) with an injection volume of 20 μL . The mobile phase consisted of 65% of 15 mM potassium phosphate buffer (pH 8.0) and 35% acetonitrile at a flow rate of 1.0 mL min^{-1} . The fluorescence signals were obtained using an excitation and emission wavelength of 355 and 460 nm, respectively (gain factor = 10). Data analysis was performed by with Agilent ChemStation. The values obtained from integration of the peak of NAT were interpolated in a calibration line, prepared using NaNO_2 (in acidic conditions) as a standard. Briefly sodium nitrite standard solutions were acidified with HCl (pH 2) in the presence of excess DAN

(0.2 mM). After 10 min, the reaction mixture was diluted in phosphate buffer at pH 7.4 (NO final concentration 1 to 80 μ M) and analyzed by HPLC.

2.2.4 Bacterial strains and growth conditions

P. aeruginosa PAO1 (ATCC BAA-47) was maintained on agar plates of Luria-Bertani medium with 10 g/L NaCl (LB10) (644520, Difco). Cultures were grown overnight in LB10 medium at 37°C with 200 rpm shaking (Infors HT, orbit diameter 25 mm). Overnight PAO1 cultures were subsequently diluted 200 times to an OD₆₀₀ = 0.05 in various culture media depending on the assay, made of M9 salts (M9S) (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, pH 7.0) supplemented with different carbon and nitrogen sources: M9GC (M9S, 0.04% w/v glucose; 0.2% w/v casamino acid), M9G (M9S, 0.4 % w/v glucose), M9-N (M9G made without any NH₄Cl), M9GCFE (M9GC, 3 μ M FeCl₃) or M9GCNO₂ (M9GC, 100 μ M to 500 μ M KNO₂) medium.

2.2.5 Growth studies

Overnight cultures of *P. aeruginosa* were washed three times in M9S made without NH₄Cl and diluted to an OD₆₀₀ of 0.05 in 200 μ l of M9GC, M9G, M9-N, M9S, M9GCFE, or M9GCNO₂ medium, which were added to each well of a 96 well plate and incubated statically at 37°C for 24 h. Furoxans were added at the time of inoculation (t = 0 h). The growth of *P. aeruginosa* was monitored spectroscopically at 600 nm, while pyoverdine production was quantified by measurement of fluorescence intensity (excitation at 398 nm and emission at 460 nm) (Tan *et al.* 2014). All measurements were carried out using a microtiter plate reader (Infinite 200 pro, Tecan). Growth rates were determined by calculating changes in OD₆₀₀ over time during exponential growth phase while growth yields refer to OD₆₀₀ values recorded at late stationary phase.

2.2.6 Biofilm assays

Overnight cultures of *P. aeruginosa* were diluted 1:200 in fresh M9 medium. One mL of the diluted culture was added into a well of a 24 well polystyrene plate (142475, Nunclon), which was subsequently incubated at 37°C with 200 rpm shaking (Infors HT, orbit diameter 25 mm) for no more than 6 h. To assess the effect of furoxan on biofilm dispersal, compounds were added into each well after 5 h incubation ($t = 5$ h) to a final concentration of 100 μ M, 200 μ M, 500 μ M or 3.6 mM, and the plates were incubated for a further 1 h. To assess the effect of each compound on biofilm formation, the furoxans were added into each well at the time of inoculation ($t = 0$ h) and biofilms were allowed to form over the next 6 h. For experiments involving NO scavengers, cPTIO was added into each well to a final concentration of 0.5 mM at the same time as furoxans. For experiments involving NO-depleted M9GC medium containing LL4212 or LL4216, 500 μ M of each furoxan were first added to M9GC medium and incubated at 37°C for 24 h prior to inoculation with *P. aeruginosa*. Biofilm biomass was quantified by crystal violet (CV) staining as described by (Barraud *et al.* 2014). The extent of dispersal was determined by the average of three independent experiments. Within each experiment, the extent of dispersal was calculated as:

% Biofilm change

$$= \left(\frac{OD_{550(control)} - OD_{550(200\mu M \text{ S150 treatment})}}{OD_{550(control)}} \right) \times 100\%$$

where a negative change is indicative of dispersal. Planktonic growth and pyoverdine production were quantified as described above (Section 2.2.5). For enumeration of colony forming units (CFU), planktonic samples were first collected in 1.5 mL Eppendorf tubes. Biofilm samples within each well were then washed twice with 1X PBS before resuspension in 1 mL of fresh PBS. Biofilm samples were subsequently obtained by physical disruption using a cell scraper (13 mm blade) and collected in 1.5 mL Eppendorf tubes. Both planktonic and

biofilm samples were sonicated in a water bath sonicator (Elmasonic PH750EL) at 37 Hz, 5 min, degas mode followed by 37 Hz, 5 min, pulse mode to minimize cell aggregation. Samples were then serially diluted, spot plated on LB10 agar plates and incubated overnight at 30°C for elucidation of colony forming units.

2.2.7 Confocal imaging and image analysis

Experiments were carried out as described (section 2.2.6) using *Pseudomonas aeruginosa* mini-Tn7-*gfp* tagged strain (PAO1-GFP) (Chua *et al.* 2016). Following treatment with LL4254, the planktonic fraction was removed and each well was washed once with PBS before being resuspended in 1 mL of fresh PBS. Images were acquired using an inverted confocal scanning laser microscope (LSM 780, Carl Zeiss). For each sample, three image stacks were obtained, accounting for an approximate area of $8 \times 10^5 \mu\text{m}^2$. Images were analyzed using the IMARIS Bitplane software (V. 8. 4.1) for quantification of the biovolume.

2.2.8 *P. aeruginosa* glucose utilization assay

The utilization of glucose by *P. aeruginosa* in M9 medium was measured using the GO assay kit (GAGO20, Sigma). Experiments were carried out as described (Section 2.2.6). Subsequently, the medium from each well was filtered and diluted in ultrapure water to obtain a glucose concentration of approximately between 20 to 80 $\mu\text{g mL}^{-1}$. Glucose standards were prepared per the manufacturer's instructions. Each volume of the standard or sample was mixed with two volumes of the assay reagent and incubated at 37°C statically for 30 min. The reaction was stopped by adding two volumes of 12 N H_2SO_4 . Glucose concentrations were determined with a microtiter plate reader (absorbance at 540 nm) and interpolating to the standard curve.

2.2.9 ATP measurements

ATP measurements were carried out using the BacTitre Glo microbial cell viability kit (G8230, Promega). Cells were grown as described (section 2.2.6) and 100 μ L of sample was collected at 6 h and mixed with equal volume of the BacTitre Glo reagent in a black opaque 96 well plate. The plate was then incubated in the dark with shaking for 5 min before the luminescence signal was recorded using a microtiter plate reader (Infinite 200 pro, Tecan). Wells filled with 100 μ L of M9GC medium were included as controls for subtraction of background signals. Relative luminescence units (RLU) were normalized to the optical density of each sample to account for increased growth of *P. aeruginosa* in the presence of 200 μ M of LL4216.

2.2.10 Statistics

All statistical tests were carried out using Graphpad Prism v7.02. Results from biofilm and growth assays were analyzed with one-way ANOVA followed by Dunnett's test for multiple comparison ($\alpha = 0.05$) against a relevant control group. Geometric means were used in statistical tests involving pyoverdine measurements and analyzed using ANOVA as described above.

2.3 Results

2.3.1 Selection of NO donors

Different classes of NO donors have different properties that govern their applicability in different contexts. For example, they may release NO either intracellularly or extracellularly. In addition, they may have different kinetics of NO release, which may be dependent on the presence of cofactors and enzymes. Further, they may show different solubility, thermal stability or stability to acids or bases. These properties in turn affect their route of administration of NO compounds, development of NO resistance, or their use as acute treatment or preventive treatments.

Due to reasons as such, it is advantageous to study the use of different NO donors under the same context. Several types of NO donors, including NONOates and nitrosothiols, had been previously studied in the context of biofilm dispersal. However, furoxans had never been evaluated for use in this process. A comparison table (Table 2.1) reflects some of the differences in these NO donors. Considering these differences and the gap in research on the use of furoxans for biofilm dispersal, we evaluated the use of furoxans-derived NO donors (furoxans) in this context using *P. aeruginosa* as our model biofilm.

Table 2.1 – Properties of different NO-donor compounds

	NONOates	Nitrosothiols	Furoxans
Chemical stability	Stable in basic solutions	-	Stable in acidic solutions
Decomposition	First order kinetics dependent on structure of nucleophilic group	Highly influenced by the environment. May be activated by transition metals, thiols, enzymes, etc	May be thiol activated or occur spontaneously
Tolerance	Lack of tissue requirement results in a lack of tolerance	Do not induce tolerance with long term use due to less stringent metabolic requirements	Lack of tolerance over long term use

Furoxans were first developed as reactive intermediates for use in organic synthesis (Katritzky and Boulton 1982, Sheremetev *et al.* 2001), and was only shown to act as NO-donors in the early 1990s by Feelish *et al.* (2009b). The release of NO under physiological conditions was thiol dependent (Feelisch *et al.* 1992b). Consequently, it was proposed that the production of different NO-redox forms (NO, nitrite and nitrate) from furoxans may involve cleavage of either the 1-2 or 3-4 bond of the furoxan ring by a thiolate anion to generate a tertiary nitroso intermediate (Feelisch *et al.* 1992b, Medana *et al.* 1994). Interestingly, it was later shown that the release of NO may also occur spontaneously in the absence of a thiol group (Sorba *et al.* 1997).

In this study, three furoxan derivatives were evaluated for their activity on biofilm dispersal via their NO-releasing properties. As biofilm dispersal response to NO is concentration dependent, fluctuations in NO release from furoxans via thiol activation could hence complicate dispersal responses. Hence, furoxans that could spontaneously release NO were chosen for the experiments. The selected furoxans were primarily phenylfuroxans. Such phenylsulfonyl substituted furoxans were amongst the first to be reported to activate soluble guanylyl cyclases from rat liver, which is suggestive of high NO activity (Feelisch *et al.* 1992b, Ghigo *et al.* 1992). LL4254, which contains an electron withdrawing substituent, $\text{SO}_2\text{C}_6\text{H}_5$, on position 3 of the furoxan ring, had also been shown to display potent effects on sGCs, aortic vasodilation and reduction of systolic and diastolic blood pressure in animal studies (Sorba *et al.* 1997). Furthermore, such sulfonyl derivatives and related compounds are water-soluble, and hence highly applicable in a physiological context (Sorba *et al.* 1997).

2.3.2 Kinetics of NO_x release from LL4254, LL4212 and LL4216

Previously, NO release from these compounds was assessed indirectly via a Griess reaction (Sorba *et al.* 1997). The Griess reaction measures the levels of nitrite, an oxidation product of NO in aqueous solution (Grisham *et al.* 1996). It is assumed that the levels of NO produced is correlated to the propensity to produce nitrite. Hence, the presence of nitrite by-products that were not derived from NO may result in an overestimation on the actual levels of NO produced. Another limitation of the Griess reaction is that it may underestimate the levels of nitrite in the presence of nitrate, a further oxidation production of nitrite. As such, the production of NO was further confirmed through reduced aortic vasodilation following the addition of oxyhemoglobin, a potent NO scavenger, in previous studies (Sorba *et al.* 1997). sGC activity, which is activated in the presence of NO, but not nitrite or nitrate, was also evaluated (Sorba *et al.* 1997).

In this study, an alternative method, the diaminonaphthalene assay, was used to evaluate NO formation. In this method, dinitrogen trioxide formed from the reaction of NO and oxygen reacts with the non-fluorescent aromatic compound 2,3-diaminonaphthalene (DAN) to give a highly fluorescent 2,3-naphthotriazole (NAT) compound that is then quantified by HPLC (Miles *et al.* 1996a). N₂O₃ is formed from a reaction of NO but not nitrite. In addition, the assay can also be conducted under physiological conditions with reduced influence from nitrite decomposition. As such, the assay offers specificity for the detection of NO. The assay also sensitive and has a low detection limit of ~10 – 30 nM N₂O₃.

The study of the kinetics of NO_x release from the furoxan compounds indicated that both NO and nitrite (NO₂⁻) were spontaneously released from the furoxans under physiologically relevant conditions (pH, temperature). The results, expressed as percent mol/mol of NO_x released with respect to the concentration of furoxan in solution (Figure 2.2a – c), showed two types of NO_x-production behavior. For LL4254, NO was the predominant species released, accounting for 82% of the total NO_x detected (~ 95%) by 24 h (Figure 2.2a); the liberation

occurred very fast, with 80% of the total NO_x produced within the first 30 min. In contrast, NO_2^- was the dominant species generated from LL4212 and LL4216 and liberation occurred in an almost linear fashion over a few hours. By 24 h, 83% and 32% of the NO_x species were detected as NO_2^- from LL4212 and LL4216, respectively, while only 2.6% and 1.2% were measured as NO (Figure 2.2b, c). An alternative chemiluminescence-based NO detection method, also confirmed the results obtained with the DAN assay (data not shown).

Previous studies showed that thiol groups such as those present in L-cysteine (L-cys) can promote NO_x liberation from furoxan compounds and increase their activity (Sorba *et al.* 1997). Since thiol groups are commonly produced by bacteria, in the form of metabolites or proteins, they are highly relevant in the context of bacterial infection treatments and their effects on NO_x release from LL4212, LL4216 and LL4254 were investigated. The exogenous addition of L-cys reduced the extent of NO_x liberated from the fast releasing compound LL4254, promoted their production from the slower releasing LL4216, but did not affect NO_x production from LL4212 (Figure 2.2d – f). At $t = 1$ h, the presence of L-cys reduced the amount of NO and NO_2^- generated from LL4254 to 4% and 36%, respectively, from the initial 70% and 90% in the absence of L-cys (Figure 2.2d). At $t = 4$ h, NO_2^- production had increased from 8% to 20% in the presence of L-cys for LL4216, while NO production from LL4216 and LL4212 was not influenced by L-cys (Figure 2.2e, f).

Figure 2.2 – Kinetics of NO release from furoxans as determined by Griess reaction and DAN method. NO (gray circles) and NOx (NO and NO₂-) (black circles) release kinetics of LL4254 (a, d), LL4212 (b, e) and LL4216 (c, f) in phosphate buffer at pH 7.4 in the absence of L-cysteine over time (a – c) and in the presence or absence of L-cysteine at selected time points (d – f). The results are expressed as percent (% mol/mol) of NO or NOx released with respect to the quantity of parent furoxan compound. Bars or symbols represent data from three or more replicates and error bars represent standard deviation from the mean.

2.3.2 Furoxan NOx donors can induce *P. aeruginosa* biofilm dispersal

To determine if the NO releasing furoxans could induce biofilm dispersal, *P. aeruginosa* biofilms were treated with 100 μ M, 200 μ M or 500 μ M of the furoxans for 1 h before quantification of biofilm biomass by CV staining. The exposure time of 1 h was selected as the maximal extent of NO release by LL4254 is achieved within 1 h. An exposure time of 1 h would also mean that biofilm can formed over the first 5 h, and reach sufficient levels required for detection of dispersal.

At 200 μ M or above, LL4254 reduced the biofilm biomass by >70% compared to untreated control samples ($p < 0.0001$) (Figure 2.3a, c). This biomass reduction, which was associated with an increase in OD₆₀₀ reading of the supernatant (data not shown), occurred after 1 h treatment, and therefore cannot be linked to growth effects but instead is a clear indication of biofilm dispersal events. These results were in agreement with previous studies, where NO was found to induce *P. aeruginosa* biofilm dispersal (Barraud et al. 2006, Barraud et al. 2009a). Dispersal by NO was also validated with 200 μ M of Spermine NONOate (S150), a NONOate with a short half-life of 39 min (Appendix B). Dispersal by LL4254 occurred in a concentration-dependent manner. In contrast, LL4212 and LL4216 were unable to induce significant biofilm dispersal at concentrations between 100 μ M and 500 μ M.

To assess if dispersal was caused by NO or other non-specific interactions between *P. aeruginosa* and the LL4254 backbone, the assay was repeated in the presence of the NO scavenger cPTIO. Under the conditions used, cPTIO alone did not have a significant effect on both planktonic and biofilm growth (data not shown). Addition of cPTIO reduced dispersal by LL4254 from an average of 73% to 18%, suggesting that the effect of LL4254 on dispersal was NO-dependent (Figure 2.3b, d). Similar results were obtained based on CFU counts from the biofilms after treatment as well as confocal imaging (Figure 2.4). Treatment with

LL4254 reduced the biofilm associated CFUs by approximately 0.5 log and by 50% based on confocal imaging. In contrast, combined LL4254 and cPTIO treatment showed biofilms that were similar in CFUs and biomass to the untreated or cPTIO treated biofilm controls.

NO release from LL4254 was established as an important factor for inducing biofilm dispersal. As the rate of both NO and NO_2^- generated from LL4212 and LL4216 was much lower than that of LL4254 under cell-free physiological conditions (Figure 2.2), it is likely that at the same compound concentration, both LL4212 and LL4216 produce a lower amount of NO. Therefore, the assay was repeated using 3.6 mM LL4212 and LL4216, which correspond to the amount of NO_x released from approximately 400 μM and 200 μM LL4254 within 1 h.

The furoxan stocks were solubilized in DMSO, and while at low concentrations ($\leq 0.5\%$ v/v) DMSO did not significantly alter *P. aeruginosa* growth or biofilm formation in our assays (data not shown), at a higher level of 3.6% v/v (the amount present when adding 3.6 mM furoxan), DMSO reduced the amount of biofilm formed by approximately 14% (Figure 2.3f). Therefore, samples to which an equivalent volume of DMSO was added, were used as a control for comparison of the extent of biofilm dispersal induced with 3.6 mM furoxan treatments. At 3.6 mM, LL4212 and LL4216 dispersed 48% and 70% of the biofilm biomass respectively (Figure 2.3e, f). Decrease in biofilm biomass observed was concomitant with an increase in planktonic biomass, thus confirming that the observed effects correlated with dispersal of bacteria from the biofilm.

Figure 2.3. – Dispersal of *P. aeruginosa* biofilms by furoxans, carried out as described in the ‘biofilm assay’. Dispersal of *P. aeruginosa* biofilms upon treatment with the furoxans for 1 h (a), or upon treatment with 200 μ M of LL4254 in the presence or absence of 0.5 mM cPTIO (b). The extent of dispersal (black circles) corresponds to an increase in OD_{600} (gray bars) and a decrease in pyoverdine fluorescence (green squares) (e). Bars or symbols represent data from three (a, b) or two (e) biological replicates whereas error bars represents the standard deviation from the mean – *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Photographs show CV stains of remaining biofilms following 200 μ M of furoxan treatment (c, d) with or without cPTIO (d) or when treated with different concentrations of furoxans (f).

Figure 2.4 – Verification of dispersal of *P. aeruginosa* biofilms by furoxans as quantified by CFU counts and confocal imaging. Enumeration of colony forming units (CFU) of biofilm (a), biovolume of biofilm based on confocal image microscopy (b) and representative confocal images (c – f) of control samples (c, e), or samples treated with LL4254 (d, f), in the presence (e, f) or absence of cPTIO (c, d). For (a), each data point represents the CFU of an independent biological replicate and error bars represent the standard deviation of the mean. For (b), each bar represents data from three image stacks and error bar represents the standard deviation.

2.3.3 Furoxans accelerate *P. aeruginosa* biofilm development and enhance glucose utilization

Because furoxans have a slower rate of release and can produce NO_x over longer periods of time, we then assessed whether these compounds can constantly prevent the switch for attachment of free-floating bacteria, maintain cells in a planktonic mode of growth and thus inhibit biofilm formation over time. Furoxans were added to M9GC medium together with the *P. aeruginosa* at the time of inoculation. After 6 h, wells that had been treated with LL4212 and LL4216 showed a significant concentration-dependent reduction in biofilm biomass, with increases in planktonic growth, compared to untreated wells, with 100 µM of LL4216 reducing biofilm biomass by > 70% ($p < 0.0001$) (Figure 2.5a, c).

To further characterize the impact of furoxans on biofilm formation, biofilm biomass of untreated control groups and groups treated with 500 µM LL4212 or 200 µM LL4216, both of which reduced the biofilm biomass at $t = 6$ h by > 80%, was quantified over time (Figure 2.5b). Surprisingly, addition of LL4212 and LL4216 promoted both biofilm formation and planktonic growth over the first 4 h and 3 h, respectively (Figure 2.5b). The levels of glucose in the biofilm cultures were then determined over time and the extent of biofilm reduction at $t = 6$ h was consistent with a reduction in glucose concentration (Figure 2.5d, e). The effect was the most pronounced with 500 µM LL4216, which led to complete glucose depletion by $t = 6$ h.

While NO is known to affect biofilm dispersal, a potential role in regulating glucose utilization and growth has not been observed before. To determine if the effects of LL4212 or LL4216 on glucose consumption were dependent on NO, biofilm prevention experiments were carried out with furoxans added together with the NO scavenger cPTIO, or using NO-depleted furoxans which had been incubated in culture medium for 24 h before inoculating with *P. aeruginosa*, thus

resulting in exhaustion of NO released from the donor compounds. The data show that after 6 h, *P. aeruginosa* biofilms had dispersed under both conditions (Figure 2.6a – c), suggesting that the effects of LL4212 and LL4216 on biofilm formation were not due to NO.

These observations of the impact of LL4212 and LL4216 on biofilms did not correlate with a typical dispersal response to NO resulting in biofilm prevention. To further elucidate the effect of the furoxans on biofilms, we then examined their impact in our assay on a known marker of biofilm dispersal in *P. aeruginosa*, including when induced by NO, which is a decrease in the production of the iron chelating siderophore pyoverdine (Chua *et al.* 2014). A similar decrease in pyoverdine levels was also previously observed upon dispersal with 200 μ M of LL4254 and 3.6 mM of LL4212 or 4216 (Figure 2.3e). The results revealed that in the presence of 500 μ M NO-exhausted LL4216, pyoverdine levels were reduced by at least 50-fold compared to untreated biofilms, even though there was no free NO present. In contrast, the use of 500 μ M of NO-depleted LL4212 had no effect on the pyoverdine levels with respect to the untreated controls (Figure 2.6c). To control for a potential direct influence of LL4216 on the pyoverdine fluorescent signal, 500 μ M of LL4216 were added to filtered, cell-free M9GC medium collected from microtiter plate cultures grown under the same conditions, which contained pyoverdine. Pyoverdine levels were found to be relatively stable in filtered medium without any compound and LL4216 at 500 μ M only slightly decreased pyoverdine relative fluorescent units (RFU) by 6.3% and 6.9%, after 6 h and 24 h respectively, compared to untreated cell-free solutions. In contrast, when 500 μ M LL4216 was added to a non-filtered culture inoculated with *P. aeruginosa* at $t = 0$ h, pyoverdine expression was > 99% lower than that of the control after both 6 h and 24 h, (Figure 2.6d).

Finally, because another major NO_x released from LL4212 and LL4216 is NO₂⁻, its effect on biofilm formation in our assay was investigated. As opposed to NO, NO₂⁻ is stable in solution and can be added directly to the culture medium. When

100 – 500 μM of exogenous nitrite were added at $t = 0$ h in place of LL4212 and LL4216, there was no significant effect on planktonic growth, biofilm formation or pyoverdine production in *P. aeruginosa* (Figure 2.7).

Figure 2.5 – Assessment of the effect of furoxans on *P. aeruginosa* biofilm formation and growth with biofilm formation assays and growth studies. Reduction of *P. aeruginosa* biofilms upon treatment with furoxans for 6 h (a), with bars representing data from three biological replicates and errors bars representing standard deviation of the mean – *, $p \leq 0.05$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Photographs show CV stains of biofilm remaining after 6 h following 200 μM furoxan treatment (c). Biofilm formation and corresponding OD₆₀₀ changes over 6 h upon addition of 200 μM of LL4216 and 500 μM LL4212 (b). Glucose concentrations were quantified at $t = 1$ h, 3 h or 6 h after addition of furoxans (d), with corresponding changes in CV staining measured (e). Symbols represent data from two biological replicates with error bars showing the standard

deviation of the mean in (e), while one representative data set was plotted for (b) and (e).

Figure 2.6 – Assessment of NO depletion of LL4212 and LL4216 to assess NO-dependent and NO-independent effects of furoxans on *P. aeruginosa* growth, biofilm formation and pyoverdine expression. *P. aeruginosa* biofilm reduction upon treatment with 500 μM of furoxans in the presence of 0.5 mM cPTIO or NO-depleted furoxans in 1-day-old M9GC medium. Bars of OD₅₅₀ measurements represent data from three biological replicates while bars of OD₆₀₀ measurements represent data from one experiment. Errors bars represent standard deviation of the mean (a). Photograph show CV stains of biofilm remaining after 6 h following furoxan treatment (b). Changes in pyoverdine levels and glucose concentrations of samples treated as described in (a), with one representative data set plotted (c). Pyoverdine changes over time in cell-free medium or culture in the presence or absence of 500 μM LL4216 (d).

Figure 2.7 – Effect of the addition of nitrite, the dominant NO_x species produced by LL4212 and LL4216, on *P. aeruginosa* biofilm formation, growth and pyoverdine production. Biofilm changes upon addition of various concentrations of KNO₂ to M9GC medium at $t = 0$ h (a) and the respective changes in planktonic growth and pyoverdine expression (b).

2.3.4 LL4212 and LL4216 are not utilized as direct carbon or nitrogen sources by *P. aeruginosa*

One possible explanation for the accelerated biofilm formation and increased growth of *P. aeruginosa* in the presence of the furoxans, is that these compounds may be used as carbon or nitrogen sources for metabolism. To determine the impact of the furoxans on metabolism, *P. aeruginosa* was inoculated in various modified M9 media with or without available carbon or nitrogen sources. In M9 medium supplemented with casamino acids and glucose as sources of carbon (M9GC), or M9 medium supplemented with glucose only (M9G), the addition of 500 μM LL4212 or LL4216 increased both the growth rates and growth yields of *P. aeruginosa* (Figure 2.8 and 2.10a), which agrees with our previous observations. In contrast, the addition of LL4212 and LL4216 to M9 medium with glucose but without any nitrogen source (M9-N), did not significantly alter growth rates, although there was a slight increase in total growth (Figure 2.8c). In M9 medium without any carbon source (M9S), *P. aeruginosa* did not grow at all whether in the absence or presence of LL4212 and LL4216 (Figure 2.8d).

Increased growth in the presence of LL4216 in M9GC medium was also associated with increased relative ATP levels quantified (Figure 2.9).

Figure 2.8 – Growth kinetics of *P. aeruginosa* with LL4212/16 in different medium for assessment of furoxans as alternative carbon sources. Growth kinetics (a– d) of *P. aeruginosa* inoculated in M9GC (a), M9G (b) or M9-N (c) or M9S (d) medium and incubated statically at 37°C.

Figure 2.9 – Measurement of relative ATP levels with LL4216 treatment using the BacTitre Glo assay, to determine effect of increased growth on ATP metabolism. Relative amount of ATP present, expressed as luminescence measured (RLU), upon treatment with 200 μM of LL4216 or equal volume of DMSO. Values expressed were adjusted for higher growth upon treatment with LL4216. Data represents measurements from two biological replicates and error bars represent standard deviation of the mean. *, $p \leq 0.05$.

2.3.5 LL4216 inhibits pyoverdine production under both low and high iron conditions in *P. aeruginosa*, but under high iron conditions reduces the growth rate

Another nutrient essential for growth in *P. aeruginosa* is iron, which is typically limiting in M9. However, bacteria can acquire essential trace amounts from contaminants in the water or components used to prepare the medium, typically by producing siderophores like pyoverdine, which has high iron affinity. Since the furoxans appeared to influence both growth and pyoverdine production in *P. aeruginosa*, we assessed if these effects may be associated with iron uptake. As expected, the addition of 500 μM LL4212 and LL4216 to M9GC resulted in increased growth and reduced pyoverdine levels (Figure 2.10b, c). In contrast, under the same conditions, LL4254 did not affect growth or pyoverdine production. In M9GC medium, the increase in growth rate showed a clear concentration dependence for LL4212, while there was only a slight increase in growth rate for LL4216 at 500 μM . With respect to pyoverdine production, LL4212 repressed pyoverdine only when added at 500 μM , while LL4216 showed a clear concentration-dependent reduction.

In M9GCFe, *P. aeruginosa* displayed an increased growth rate of about 8-fold compared to M9GC. In M9GCFe, LL4212 addition resulted in a slight reduction in growth rate relative to the control while LL4216 reduced the growth rate in a concentration dependent fashion, with a 38% reduction when added at 500 μM (Figure 2.10b). The presence of added iron also generally reduced pyoverdine production. While LL4212 only slightly further reduced this production, by 12% when added at the highest concentration tested, the effects were more pronounced with LL4216, which at 500 μM induced a drastic further reduction in pyoverdine to background levels. The addition of LL4254 had no influence on growth rate and pyoverdine production in either medium (Figure 2.11). Taken together these data suggest that LL4212 and LL4216 may have opposite impacts on *P.*

aeruginosa growth depending on the availability of iron, resulting in increased growth under iron limited conditions, while reducing growth when iron is replete.

Figure 2.10 – Growth rates and pyoverdine production of *P. aeruginosa*, in the presence of furoxans, in different medium with or without iron supplementation. Growth rates in the absence or presence of 500 μM of LL4212, LL4216 and LL4254, calculated by changes in OD₆₀₀ during the exponential growth phase, in M9 medium supplemented with different carbon or nitrogen sources (a). Growth rates (b) and pyoverdine fluorescence (c) at 24 h of *P. aeruginosa* inoculated statically at 37°C in M9GC (Fe³⁺-) or M9GCFe (Fe³⁺+) medium in the presence of 100 – 500 μM of the furoxans. Bars represent data from three biological replicates and errors bars representing standard deviation of the mean. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

Figure 2.11 – Measurement of growth and pyoverdine expression over 24 h to determine the relation between pyoverdine levels, iron availability and growth with furoxan addition. Growth kinetics (a, c, e) and pyoverdine expression (b, d, f) of *P. aeruginosa* grown statically at 37°C with furoxans in the absence (black) or presence (orange) of Fe³⁺, over 24 h.

2.4 Discussion

NO has been implicated as a signal for biofilm dispersal in several bacterial species, and has been suggested as an alternative strategy for the control of bacterial biofilms (Barraud *et al.* 2015). The use of NO, however, is often limited by its range of effective concentration and its specificity towards other biological processes (Miller and Megson 2007, Moncada *et al.* 1991b, Thomas *et al.* 2008b). Different classes of NO donors confer flexibility and choices in terms of the rate and duration of NO release, as well as modifications to enable site-specific NO-release (Barraud *et al.* 2012, Miller and Megson 2007). In this study, the suitability of furoxan-derived NO donor compounds as biofilm dispersal agents were evaluated in *P. aeruginosa* biofilms. These compounds were found to induce biofilm dispersal via NO release as well as influence *P. aeruginosa* growth and biofilm development in a NO independent manner, and reduce pyoverdine levels through both NO-dependent and NO independent means.

NO_x release from furoxans was evaluated using an alternative DAN method that enables NO release to be distinguished from NO₂ release (Fang *et al.* 2009, Miles *et al.* 1996b), as compared to the previous study in which only NO₂ was measured (Sorba *et al.* 1997). The relative rates of NO release were consistent with previously published results, in which total NO_x measured were highest in LL4254 > LL4212 > LL4216 (Sorba *et al.* 1997). Interestingly, while ~80% of NO_x released from LL4254 were detected as NO, NO accounted for only 3 – 5% of total NO_x measured from LL4212 and LL4216 (Figure 2.2). Proposed mechanisms of furoxan activation indicate that NO or NO⁻ formation preludes the formation of NO₂ and NO₃, which are formed following oxidation of NO in aqueous solutions (Gasco and Schoenafinger 2005, Medana *et al.* 1994, Sorba *et al.* 1997). This oxidation process follows second order kinetics (Ford *et al.* 1993, Ignarro *et al.* 1993). Hence, in the absence of biological tissues, at higher concentrations (> 300 μM), NO is predicted to have a short half-life of <1 s

(Ignarro *et al.* 1993). A fast rate of oxidation may explain the presence of low amounts of NO₂ in LL4254 containing samples, while NO, released from the parent furoxan, remains the predominant species in solution. Similarly, due to second order kinetics, lower concentrations (0.05 – 1 µM) of NO have a half-life of 500 s to a few hours (Ignarro *et al.* 1993). As such, LL4212 and LL4216, which have a lower steady state level of NO, is expected to have a slower conversion to NO₂ and higher proportion of NO as compared to NO₂. However, this was not observed and suggests that NO release from LL4212 and LL4216 may follow different mechanisms as compared to LL4254. This is beyond the scope of the study as, to date, the exact mechanism through which furoxans are activated to generate NO bioactivity have not been defined, but is instead known to be dependent on the substituent groups present on the furoxan ring (Friedrichsen 2001, Gasco and Schoenafinger 2005, Sorba *et al.* 1997).

Although the mechanism of activation of furoxans is unclear, the relative extent of NO generation does not change amongst the evaluated compounds, with highest rate and levels of NO released from LL4254 > LL4212 > LL4216. The furoxan, LL4254, which has a fast decomposition rate, dispersed *P. aeruginosa* biofilms via NO when used at concentration of >200 µM. This is consistent with previous studies that showed that NO mediates biofilm dispersal (Barraud *et al.* 2006, Barraud *et al.* 2009b), and that micromolar concentrations of NONOates were efficient at removing biofilm biomass (Barnes *et al.* 2013, Barnes *et al.* 2015, Marvasi *et al.* 2014). At the same concentration, LL4212 and LL4216 did not have significant effects on the biofilm biomass. This was not unexpected given that LL4212 and LL4216 has a much slower, but prolonged release of NO. While the steady-state concentration of NO is not measured in this study, one would expect that slower rates of NO release reduces effective NO concentration perceived by the bacteria. This is because NO generated may be consumed by cellular processes, reaction with oxygen, and reaction with other chemical

compounds present in the medium (Miller and Megson 2007, Thomas *et al.* 2008a). Similarly, NO-donor compounds used in eukaryotic systems had also been shown to display different potencies with regards to endothelial cyclic GMP accumulation, with effective concentrations of the NO donor positively correlating to their rates of NO release (Ferrero *et al.* 1999).

An increase in both LL4212 and LL4216 concentration used (to 3.6 mM) dispersed the biofilm. Despite having a lower rate of NO release, high concentrations of LL4216 had a greater dispersal effect than equimolar of LL4212. This may be due to difference in compound activation in the presence of thiol groups that are produced or secreted by the bacteria (Gasco and Schoenafinger 2005, Sorba *et al.* 1997). NO release from the donor SNAP, which is similarly dependent on the presence of reduced thiols and transition metals, had also been reported to produce different concentrations of NO when used at the same concentration, depending on cell type and medium composition used (Ferrero *et al.* 1999, Matthews *et al.* 1996, Singh *et al.* 1996). Dispersal using higher levels of LL4212 or LL4216 corresponded with a 'reduced pyoverdine' phenotype indicative of the presence of increased NO (Barraud *et al.* 2006), suggesting that slow NO-releasing donors could also be used to mediate dispersal when used at sufficiently high concentrations. The results hence suggest that furoxans with a fast or slow NO release profile could be used at low or high concentrations respectively, to promote biofilm dispersal. In addition, slow NO-releasing furoxans were found to be potent at preventing biofilm formation over longer periods of time, in a manner similar to the prevention of *P. aeruginosa* biofilm formation by other slow NO releasing polymers (Duong *et al.* 2014). This suggests that slow NO-releasing furoxans may be useful at higher concentrations to induce dispersal and prevent re-formation of biofilms over time. However, it should be noted that a potential role of NO in mediating the effects of furoxans on biofilm prevention could not be clearly determined here, and biofilm

prevention may be a function of the furoxan backbone or decomposition product instead of NO.

In addition to their roles on biofilm dispersal, LL4212 and LL4216 were found to affect biofilm development over time. The two furoxans initially increased planktonic growth and biofilm formation relative to the control, but subsequently caused premature biofilm dispersal with continued planktonic growth. A similar sharp decrease in biofilm biomass during growth had been observed in *P. aeruginosa* biofilms and was linked to a sudden depletion of the carbon source and the onset of starvation (Schleheck *et al.* 2009). In support of this, glucose concentrations were shown to decrease rapidly in samples treated with furoxans in a concentration dependent manner. The results hence suggest that LL4212 and LL4216 increase the growth rates of *P. aeruginosa* through increased rates of glucose metabolism and cause dispersal via a carbon or glucose starvation-induced response (Gjermansen *et al.* 2005, Huynh *et al.* 2012). The use of NO depleted LL4212 or LL4216 in M9GC medium induced the same effects. Addition of nitrite, the main NO_x species released from LL4212 and LL4216, did not affect planktonic growth, biofilm formation, or pyoverdine production, suggesting that the backbone or by-product of NO_x release from these two compounds, but neither NO or NO₂⁻, were responsible for the increased growth.

Subsequent growth studies showed that LL4212 and LL4216 increased growth rates and total yield relative to the untreated control groups. One of the factors that promote bacteria growth is higher nutrient levels, and the availability of dissolved organic material or addition of aromatic compounds had been show to promote growth in cases where carbon sources are limiting (Lechevallier 2003, Lindell *et al.* 1995). However, in the absence of carbon or nitrogen source, both furoxans could not support growth. Instead, increased utilization of glucose and higher ATP levels were only observed in furoxan treated samples grown with sufficient energy sources. Another factor that promotes bacteria growth indirectly

is the presence of trace minerals and growth factors, such as iron and vitamins, which are essential cofactors of proteins regulating metabolic processes (Messenger and Barclay 1983). While there is no evidence that LL4212 or LL4216 or their decomposition products act as cofactors, it is possible that they could play a similar, indirect role to promote growth by interfering with processes related to glucose metabolism, ATP production, or NADH/NAD⁺ turnover. Alternatively, increase glucose uptake and ATP production may be an outcome, but not cause of increased growth. LL4212 and LL4216 may induce a rapid growth and transition into the exponential phase through unknown mechanisms. Higher ATP levels measured could then simply be due to its increased secretion during exponential growth phase, a process which had been observed in *P. aeruginosa* and other bacteria grown in glucose (Hironaka *et al.* 2013, Schneider and Gourse 2004). Lastly, bacteria have been shown to display increased growth when subjected to stress, such as growth in the presence of antibiotics. Such resistant strains were obtained following several generations of adaptation and evolution in sub-inhibitory antibiotic concentration (Reding-Roman *et al.* 2017). Increased growth rates and yield observed with antibiotic stress is also preceded by a longer lag phase (Reding-Roman *et al.* 2017). No increases in lag phase duration were observed with LL412 or LL4216 addition. Addition of LL4212 and LL4216 at the concentrations tested was also non-toxic to *P. aeruginosa*. As such, the increased growth observed is not likely due to a stress response by the bacteria.

Besides having an effect on growth and biofilm development, LL4212 and LL4216 also affected levels of pyoverdine, a siderophore produced by *P. aeruginosa* with a high affinity for Fe³⁺ (Cox and Adams 1985). The decrease in pyoverdine levels upon LL4212 and LL4216 addition may be in part due to NO release, which is known to repress the expression of pyoverdine related genes (Barraud *et al.* 2009a). Indeed, when NO-depleted LL4212 was used, no repression in pyoverdine production was observed. In contrast, the effect of

LL4216 on pyoverdine appeared to be independent of NO, as the NO-depleted compound still resulted in a decrease in pyoverdine levels. Overall, the results show that decreases in pyoverdine production with LL4212 are predominantly mediated by NO while similar decreases in LL4216-treated samples were either due to the presence of NO or mediated directly by the LL4216 backbone or its decomposition products. Despite these differences, both compounds were able to promote bacterial growth in *P. aeruginosa* and no correlation between growth rates and pyoverdine levels was observed. Thus, the furoxans likely exert independent effects on growth and pyoverdine production.

Figure 2.12 – Relationship between the furoxan compounds, pyoverdine levels, and bacterial growth. All furoxan compounds can release NO to different extents. NO then inhibit pyoverdine production. Pyoverdine levels are important during growth in iron limited conditions, where they act as iron scavengers. Consequently, pyoverdine levels are reduced in the presence of iron. The presence of iron also promotes bacterial growth up to a certain extent, but may cause toxicity at higher levels. Interestingly, LL4216 inhibits pyoverdine levels, but promote growth under iron limited conditions, while reducing growth with increased iron availability. LL4212 also inhibits pyoverdine production, but do so through the activity of NO, as the addition of cPTIO abolishes this effect. Independently, LL4212 also promotes growth through unknown mechanisms.

It was interesting to note that LL4216 was able to simultaneously promote growth and repress pyoverdine levels when *P. aeruginosa* was grown in minimal medium limited in iron. This is because micromolar concentrations of iron are generally needed for bacterial survival and growth (Guerinot 1994, Poole and McKay 2003), and pyoverdine is one of the main systems employed by the bacteria to sequester the metal (Guerinot 1994). Initially, we hypothesized that LL4216 may function as a siderophore in place of pyoverdine. Repression of pyoverdine production by LL4216, while still being able to uptake iron, could then direct metabolites towards energy generation and growth, and away from the costly production of pyoverdine, which has been previously estimated to require approximately 10% of additional carbon consumption per growth unit in a closely related species, *Pseudomonas putida*, grown under iron limitation (Hider and Kong 2010, Sasnow *et al.* 2016). Under this scenario, the presence of LL4216 in medium supplemented with iron should further promote growth by enabling higher iron uptake. Contrary, a reduction in growth, as compared to the untreated sample, was measured (Figure 2.10). In addition to their roles in growth, iron also plays a dual role as a scavenger of free radicals generated in oxidative or nitrosative stresses or as a catalyst for the formation of these radicals (Koskenkorva-Frank *et al.* 2013). It is possible that increased uptake of iron, should LL4216 function as a siderophore, had negative effects on growth by affecting the balance of such redox regulation. However, iron was supplemented at a low concentration of 30 μM in the experiments, compared to concentrations of 50 – 300 μM iron (III) chloride commonly used in iron-replete medium in experiments involving *P. aeruginosa*. In these experiments, iron supplementation was able to promote *P. aeruginosa* growth and biofilm formation (Banin *et al.* 2005, Oglesby *et al.* 2008a). Furthermore, a much higher concentration of iron at $>400 \mu\text{M}$ was required to induce toxicity in other relevant *Pseudomonas spp* (Kim *et al.* 2009). In addition, low rates of NO release from LL4216 suggests low levels of effective NO concentration in solution. This, together with the lack of toxicity observed with LL4216 addition, indicates that addition of LL4216

likely does not cause nitrosative stresses for the bacteria. As such, LL416 likely does not function as an alternative iron scavenger, but instead exert growth-related effects independent of pyoverdine production and iron acquisition.

Conclusions and Future studies

In this study, it was found that appropriate concentrations of furoxans could be used as biofilm dispersal agents. LL4254 was the most effective at inducing *P. aeruginosa* biofilm dispersal. Given the different properties and advantages that furoxans have over other NO-donors, the role of LL4254 as a biofilm dispersing agent could be further investigated in other species.

Besides inducing biofilm dispersal via NO, the furoxans LL4212 and LL4216 also influenced growth and pyoverdine production of *P. aeruginosa*. The data suggests that the two furoxans exert effects on growth and pyoverdine levels independent of each other. In addition, further preliminary studies indicate that the furoxan could promote growth in various bacteria, including those that do not produce pyoverdine, to different extents (Figure 2.12). Further studies are required to understand the mechanism of action of the furoxans. For this purpose, furazans analogs may be used to distinguish between the effects exerted by NO and the furoxan backbone or its decomposition products. The analogs are structurally similar to furoxans, but have different redox behaviours and do not release NO (Figure 2.13), had been used to assess the involvement of NO in similar contexts (Bertinaria *et al.* 2003).

Figure 2.13 – Growth of PAO1, *P. putida*, *P. fluorescens* (Pf5), *E. coli* and *S. aureus* in the presence of 500 μ M of LL4216 in M9GC medium.

Figure 2.14 – General structure of furoxans (a) and the furazan analogs (b)

LL4212 and LL4216 resulted in the growth and early dispersal of biofilms through glucose depletion. It would hence be of interest to evaluate if continuous supplementation of glucose would further promote planktonic and biofilm growth, or would still lead to biofilm dispersal over time. This could be carried out in a flow system, where glucose can be continuously provided. The outcome of such study would determine if LL4212 and LL4216 would be suitable for use as extended NO release dispersal agents, where consistent increase in bacteria growth would not be a desirable outcome. Alternatively, such furoxans compounds could be useful in biocultures and biotransformation processes, where faster growth would be useful for the production of biomolecules or removal of toxic compounds (Pitt and Ross 2003).

Lastly, it was shown that LL4216 represses pyoverdine production in *P. aeruginosa*. In addition to the use of furazan analogs, the role of LL4216 could be looked into through the use of RT-PCR that targets genes regulating the pyoverdine synthesis process

3. Cephalosporin-linked NO donor prodrugs

3.1 Introduction

Nitric oxide (NO) is a highly reactive molecule that plays important roles in the physiology or pathology of biological processes, such as in immune responses, nervous systems and endothelial functions (Bogdan 2001b, Moncada *et al.* 1991a, Witte and Barbul 2002). In addition, NO is a free radical that can react with other compounds and chemical groups, such as oxygen, superoxide ions and amine groups to generate nitrogen dioxide, peroxynitrite, and S-nitrosothiols, which may act as irritants, oxidizing agents or contribute to nitrosative stresses (Thomas *et al.* 2006). Due to various interactions between NO and different targets, the use of available NO donors that spontaneously releases NO may result in non-specific effects. Furthermore, the spontaneous nature of these NO donors limits the delivery of NO to a biofilm in both clinical or industrial settings. A targeted approach of NO-delivery may hence enable the use of lower amounts of NO donors, reducing NO toxicity and off target effects.

Figure 3.1 – Schematic of the mechanism of activation of cephalosporin-linked NO donor pro-drugs by β -lactamases and subsequent NO release from the diazeniumdiolate component for biofilm dispersal. Figure is reprinted from Barraud *et al.* (2012) with permission from John Wiley and Sons.

For this purpose, cephalosporin-linked NO donor pro-drugs have been developed for NO delivery to the site of infection. These pro-drugs are hybrid compounds consisting of a cephalosporin backbone linked to a diazeniumdiolate, or NONOate group (Section 1.5). These compounds have been designed for activation and release of NO only in the presence of β -lactamases, by making use of the disposition of cephalosporins to eject leaving groups from the 3' position following cleavage of the β -lactam ring (Yepuri *et al.* 2013). The free NONOate is then able to spontaneously release NO under physiological conditions in accordance to the chemistry of its amine side chains (Figure 3.1). The use of cephalosporin as a component to stabilize the NONOate prior to pro-drug activation is viable as β -lactamases are bacteria specific, with no mammalian homologues present for activation of the compound. The rise of β -lactam resistant and β -lactamase producing bacterial strains also suggest that commonly isolated clinical bacteria can readily activate the pro-drug (Livermore 2009, Zeng and Lin 2013). In addition, β -lactamases are known to accumulate around biofilms, which can be dispersed by low concentrations of NO (Barraud *et al.* 2006). By selecting NONOates with short half-lives and fast NO release kinetics, it is thought that diffusion of NO would occur primarily around the biofilm, reducing non-specific interaction of NO with other moieties. Such cephalosporin-linked NO donor pro-drugs have been studied *in vitro* and tested against *P. aeruginosa* and *Haemophilus influenzae* biofilms, where they were found to promote *P. aeruginosa* biofilm dispersal and antibiotic susceptibility of *P. aeruginosa* towards ciprofloxacin and tobramycin and *H. influenzae* towards azithromycin (Barraud *et al.* 2012, Collins *et al.* 2017).

β -lactams are the most commonly used antibiotic, accounting for > 50% of global sale of antibiotics (Hamad 2010). They function by binding to and inhibiting penicillin binding proteins (PBP/ DD-transpeptidases), which are involved in trans-peptidation and crosslinking of peptidoglycan layers during cell wall synthesis. This consequently leads to disruption of cell wall synthesis, loss of

selective permeability, cell lysis and cell death (Zeng and Lin 2013). Resistance to β -lactams may arise due to several factors and had been extensively reviewed (Fuda *et al.* 2004, Jacoby 2009, Kohler *et al.* 1999, Korfmann and Wiedemann 1988). Of which, the production of β -lactamases that cleaves the β -lactam ring and inactivate the antibiotic remains one of the most important modes of resistance towards β -lactam drugs and is the most common mechanism of β -lactam resistance amongst gram-negative bacteria, including *P. aeruginosa* (Jacoby and Munoz-Price 2005, Zeng and Lin 2013).

In the case of β -lactamase mediated resistance, the *P. aeruginosa* genome encodes several β -lactamases, including the chromosomally inducible β -lactamase (*ampC*). Induction of *ampC* is the most common amongst penicillin and cephalosporin resistant strains of *P. aeruginosa*, and has been reported in up to 56% of patients treated with β -lactam drugs (Wolter and Lister 2013). Further, ~65% of *P. aeruginosa* isolates displaying increased tolerance or resistance to carbapenams, a β -lactam prescribed for infections known or suspected to be caused by multidrug-resistant bacteria, had been shown to express extended spectrum β -lactamase resistance (Rodriguez-Martinez *et al.* 2009). This resistance displayed by *P. aeruginosa* strains make them a viable model for the study of the cephalosporin-linked NO-donor prodrugs in this study.

In this chapter, six cephalosporin-linked NO donor compounds were evaluated for their applications using mouse infection models for proof-of-concept studies. NO release and activity from these compounds had been previously characterized, but their use *in vivo* had not been investigated (Barraud *et al.* 2012, Collins *et al.* 2017, Yepuri *et al.* 2013). For this purpose, NO release and activity were again evaluated to ensure batch-to-batch consistency. In addition, biological properties that had not been previously characterized, such as the minimum inhibitory concentrations (MIC) of the hybrid drugs and the toxicity of the lead compound against a mammalian cell line, were evaluated. Lastly, experiments were carried

out to evaluate how factors, such as the extent of β -lactamase induction, may influence NO release from the compounds, to optimize conditions for animal testing.

The prodrugs were found to show variable efficacy in different animal models, which was in part due to the challenge of activating the prodrug with imipenem. Overall, the results indicate that such prodrugs, when used alone or in combination with antibiotics, are a promising approach to controlling biofilm-based infections. However, the three-step-activation of the compound, induction of β -lactamase, followed by cleavage of the hybrid compound by β -lactamase, and finally, release of NO from the NONOate component, may pose a challenge for optimization of their use and dosage. Future studies will hence focus on the development new candidates that display specificity to activation by PBPs to circumvent this problem.

3.2 Materials and methods

3.2.1 Cephalosporin prodrugs

Figure 3.2 – Structures of the cephalosporin-linked diazeniumdiolate (NONOates) NO donor pro-drugs. The blue segments represent different side groups of the cephalosporin component while the black segment is the cepham core containing a β -lactam ring present in all β -lactam drugs. The red segments represent different NONOates, namely DEA NONOate (a, d, f), proli-NONOate (b), phenylpiperazine (Pen-pip NONOate) (c), and pyrimidinepiperazine (Pyr-im-pip NONOate) (e). All compounds were provided by M. Kelso of the University of Wollongong.

Cephalosporin-linked NO donor compounds used in this study are listed in Table 3.1 with their respective structures presented in Figure 3.2. The compounds were synthesized as described in part in Barraud *et al.* (2012) and Yepuri *et al.*, (2013) and provided by M. Kelso of the University of Wollongong. Cephalosporin-linked NO donor compounds were dissolved in DMSO to a stock concentration of 100 mM and stored at -20°C . Similarly, Spermine NONOate (S150) (82150, Cayman chemical) was dissolved in 10 mM NaOH and stored at -20°C . β -lactam based antibiotics, imipenem monohydrate (IMIP) (I0160, Sigma Aldrich) and ceftazidime (A6987, Sigma Aldrich) were prepared in milliQ water fresh before each experiment and kept at 4°C for no longer than one day before use. Colistin sulphate (Col) (C4461, Sigma Aldrich) was prepared in milliQ water and stored at 4°C .

Table 3.1 – Compound ID and half-life of NONOate component of cephalosporin-linked diazeniumdiolates.

Compound ID	Cephalosporin	NONOate	NONOate T _{1/2}
UW0 (DEACP)	Cefaloram	DEA NONOate	2 min
UW1	Cefaloram	DEA NONOate	2 min
UW2	Cefaloram	Proli NONOate	3 s
UW3	Cefaloram	Pen-pip NONOate	2 min
UW4	Cephalosporin 2	DEA NONOate	2 min
UW5	Cefaloram	Pyrim-pip NONOate	1.9 min
UW6	Cephalosporin 3	DEA NONOate	2 min

3.2.2 Bacterial strains and culture conditions

P. aeruginosa strains used are listed in Table 3.2. All strains were routinely cultured in LB10 medium (644520, Difco) and maintained on LB10 agar plates (244520, Difco). Unless otherwise stated, overnight cultures were obtained by inoculation in 10 mL LB10 medium in a 50 mL falcon tube with incubation at 37°C at 200 rpm for 16 – 18 h. M9 medium used consisted of 1 × M9 salts (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, pH 7.0) was supplemented with 0.04% glucose and 0.2% casamino acids.

Table 3.2 – List of strains used in this study

Strain	Genotype	Source
<i>Pseudomonas aeruginosa</i> PAO1	Wildtype	ATCC BAA-47
MPAO1	Wildtype	(Jacobs et al. 2003)
PAO1 <i>nirS::gfp</i>	<i>nirS::gfp</i>	(Barraud et al. 2006)
MPAO1 <i>fhp-F06::ISlacZ/hah</i>	<i>fhp-F06::ISlacZ/hah</i>	(Jacobs et al. 2003)

3.2.3 Evaluation of minimum inhibitory concentration of antibiotics

The minimum inhibitory concentration (MIC) was determined through the broth microdilution method as described by Wiegand (2008). Briefly, overnight

cultures of *P. aeruginosa* PAO1 were sub-cultured in fresh medium, incubated at 37°C with 200 rpm shaking and collected during mid-log phase ($OD_{600} = 0.4 - 0.6$). Cultures were then washed and re-suspended to an OD_{600} of ~ 1.0 ($\sim 1.0 \times 10^9$ per mL). A working stock of bacteria was made by further diluting the suspension $1000 \times$ to $\sim 1.0 \times 10^6$ CFUs per mL.

One hundred μ L of Mueller Hinton broth (MHB) was first added into each well of a 96 well plate. MHB is commonly used for broth microdilution methods of antimicrobial susceptibility testing as it is a rich medium with low amounts of antagonists and meets the CLSI standard requirements. An additional 100 μ L of Mueller Hinton broth (MHB) was then added to the sterile control wells, while 100 μ L of the bacteria suspension was added to the growth control wells to a final CFU of $\sim 5.0 \times 10^5$ cells/mL. To a column of the remaining wells, 100 μ L of stock antibiotics was added and serially diluted two-folds across the remaining columns to a final concentration of 0.25 – 256 μ g/mL. One hundred μ L of the bacteria suspension was then added to each of the antibiotic-containing wells to a final CFU of $\sim 5.0 \times 10^5$ cells/mL. The plate was incubated statically at 37°C with growth monitored every 20 min through measurement of absorbance at 600 nm using a microtitre plate reader (Infinite 200 pro, Tecan). The minimum inhibitory concentration of each antibiotic was defined as the concentration in which no change in OD_{600} was observed over time. The minimum biocidal concentration (MBC) was evaluated by streaking out cultures from each well on LB10 agar and incubating at 37°C overnight. MBC was defined as the concentration at which no visible growth was observed following incubation.

3.2.4 Biofilm plate dispersal assay (24 well)

Overnight cultures of *P. aeruginosa* were diluted 200 times in fresh M9GC medium. One mL of the diluted culture was added into a well of a 24 well polystyrene plate (142475, Nunclon), which was subsequently incubated at 37°C with 200 rpm shaking. Unless otherwise stated, to assess the effect of the

cephalosporin-linked NO donor prodrugs on biofilm dispersal, imipenem was first added into each well at $t = 5$ h to a final concentration of $1 \mu\text{g/mL}$. At $t = 6$ h, each of the NO donor prodrugs were added into each well to a final concentration of $100 \mu\text{M}$, $200 \mu\text{M}$ or $500 \mu\text{M}$ and the plates were further incubated for another 15 – 30 min. When the spontaneous NO donor, S150 was used, the compound was added to a final concentration of $200 \mu\text{M}$ at $t = 6$ h for 15 – 30 min.

Biofilm biomass was quantified by crystal violet (CV) staining (Barraud *et al.* 2014) and dispersal was evaluated as a percentage of biofilm change as described in chapter 2. CFU counts were also carried out as described in Chapter 2, section 2.2.6. Results from plate biofilm assays were analysed with one-way ANOVA followed by Dunnett's test for multiple comparison ($\alpha = 0.05$) against the relevant control group in Graphpad prism V7.02

3.2.5 Confocal microscopy

Confocal imaging was carried out as described in section 2.2.7. For each sample, at least three image stacks were obtained, accounting for an approximate area of $8 \times 10^5 \mu\text{m}^2$. Images were analyzed using the IMARIS Bitplane software (V. 8. 4.1) for quantification of the biovolume. Results from the software analyzed with one-way ANOVA followed by Dunnett's test for multiple comparison ($\alpha = 0.05$) against the relevant control group in Graphpad prism V7.02.

3.2.6 Quantification of NO release from the NO prodrugs

NO release from the cephalosporin-linked NO donor prodrugs was measured using a free radical analyser (TBR 1025, World Precision Instruments) consisting of a 2 mm nitric oxide sensor probe (ISO-NOP, WPI) placed within a stainless steel outer sleeve (#5436, WPI) and pressed against a polymeric membrane

(#5436, WPI). The interior of the probe was filled with an electrolyte filling solution (#7325, WPI).

Data output from the free radical analyser was measured in real time via the LabScribe3 software. Data output in pA was converted to molarity of NO detected based on the instrument sensitivity of 2 pA/nM. The amount of NO released was calculated as stated:

$$[NO](nM) = \frac{pA_{output} - pA_{baseline} (pA)}{2 \frac{pA}{nM}}$$

Where the baseline refers to the stable output obtained when the NO sensor probe is placed in the buffer before the addition of any NO donor compounds.

All NO measurements were carried out in 10 mL of M9 salts at room temperature with 500 μ M of the NO donor compound. When required, 50 μ L of penicillinase (P0839, Sigma Aldrich), equivalent to 0.1 KU of enzyme, was added into the solution. Between each reading, the probe was immersed briefly (~10 s) in 0.1 M HCl and Tergazyme solution (Z273287, Sigma Aldrich) to remove any organic matter or protein build-up on the polymeric membrane. Rates of NO release were determined through linear regression for data points lying between $t = 7 - 10$ min for UW-0 – 1 and UW-3 – 6 and between $t = 4 - 6$ min for UW-2.

3.2.7 Quantification of β -lactamase activity

β -lactamase activities of bacterial cells following treatment with imipenem, the cephalosprin-linked NO donor compound, DEACP, N-acetylmuramic acid (NAM) (A3007, Sigma Aldrich) and N-acetylglucosamine (NAG) (A8625, Sigma Aldrich) were quantified using a β -lactamase activity assay kit (MAK221, Sigma Aldrich). Treatment of *P. aeruginosa* with the compounds were carried out as described above (Section 3.2.4). Measurements and calculations were carried out as per the manufacturer's instructions.

The reaction mix was made by adding 2 μL of nitrocefin to 48 μL of β -lactamase assay buffer. Controls in which no nitrocefin was added were also included. Equal volumes (50 μL) of reaction mix or the control reaction mix were then added to 50 μL of sample in each well of a 96-well plate. Absorbance at 490 nm (Abs_{490}) was then measured using a microtitre plate reader (Infinite 200 pro, Tecan), with one reading taken every minute for at least 30 min.

The standard curve (0 – 20 nmole/well) was generated by adding 0 – 10 μL of hydrolysed 2 mM (2 nmole/mL) nitrocefin standard solution into each well and bringing the volume to 100 μL with the β -lactamase assay buffer.

The β -lactamase activity is calculated based on the following formula:

$$\beta - \text{lactamase activity (nmole/min)/mL} \\ = \frac{Abs_{490_2} - Abs_{490_1} \left(\frac{\text{nmole}}{\text{well}} \right)}{(t_2 - t_1)(\text{min}) \times \text{sample volume} \left(\frac{\text{mL}}{\text{well}} \right)}$$

where the concentration of hydrolysed nitrocefin at $t = 1$ and 2 (Abs_{490_1} and Abs_{490_2}) were determined based on standard curves generated at the same time point. β -lactamase activity with imipenem treatment were analysed with one-way ANOVA followed by Tukey's test for identification of significant differences between sample means ($\alpha = 0.05$). Activities from three independent experiments were used for analysis in Graphpad prism V7.02.

3.3.8 Cell culture and assessment of compound toxicity

A H1299 (CRL-5803, ATCC) lung epithelial cell line was used for assessment of compound cytotoxicity and was maintained in DMEM medium (11965118, Thermo fisher) containing 10% heat inactivated fetal bovine serum (FBS) (10500064, Thermo fisher) at 37°C and 5% CO_2 .

Compound toxicity was evaluated using a LDH cytotoxicity assay kit (88953, Pierce). 100 000 H1299 cells were seeded in 96 well plates in 100 μL of DMEM

medium and allowed to attach to the surface overnight (14 – 16 h). Subsequently, cells were resuspended in 100 μ L of fresh DMEM medium and treated with 100 – 1000 μ M of DEACP, equivalent concentrations of DMSO (0.1 – 1%), milliQ water or 1 $\times$ lysis buffer for 45 min. Fifty μ L of the sample medium was then mixed with 50 μ L of the reaction mixture and incubated at room temperature for 30 min in the dark. The reaction was stopped via the addition of 50 μ L of stop solution and the absorbance at 490 nm and 680 nm was measured using a microplate reader and the % cytotoxicity was determined as:

$$\frac{(Abs_{490} - Abs_{680})_{Compound} - (Abs_{490} - Abs_{680})_{Water}}{(Abs_{490} - Abs_{680})_{Lysis} - (Abs_{490} - Abs_{680})_{Water}} \times 100\%$$

The optimal cell number for use in the LDH assay was evaluated by seeding 0 – 160,000 cells per well and carrying out the experiment with 1 $\times$ lysis buffer as described above. The LDH signal ($Abs_{490} - Abs_{680}$) was plotted against cell number and a cell number within a linear range of LDH signal was selected for use in further experiments.

3.2.10 *In vitro* studies of factors affecting dispersal in mouse models

3.2.10.1 Modification of biofilm plate dispersal assay

Biofilm plate dispersal assays were carried out as described (Section 3.2.4). Modifications were made depending on the factors tested. To evaluate the effects of duration of β -lactamase induction, imipenem was either added in at different times ($t = 5$ h, 5 h 15 min, 5 h 30 min or 6 h) or excluded, while DEACP was added in at $t = 6$ h for 15 – 30 min as previously described. To evaluate the effects of washing on the ability of the compounds to disperse biofilms, the medium in each well was replaced with fresh M9 medium before addition of 200 μ M of DEACP (1X wash) or before the addition of 1 μ g/mL of imipenem and 200 μ M DEACP (2X wash). To evaluate the effects of multiple doses of NO donor treatment on biofilm dispersal, experiments were carried out as described

(Section 3.2.4), with the addition of S150 to a final concentration of 0, 20 or 200 μM at $t = 2$ h and 4 h and 200 μM at $t = 6$ h. Washing steps were also included before each treatment at $t = 2, 4$, and 6 h or $t = 6$ h or 15 min after each treatment at $t = 2$ h 15 min and 4 h 15 min.

3.2.10.2 Evaluation of biofilm dispersal from implants

Silicone tubes with 4 mm interior diameter, 1 mm thickness and 3 mm length (implants) were sterilized by soaking in 70% ethanol for at least 1 hour before air drying and exposure to UV for 15 min. Overnight cultures of *P. aeruginosa* MPAO1 were washed two times by centrifugation at $10\,000 \times g$ and resuspension of the pellet in 1 mL of $1 \times \text{PBS}$. The implants were then suspended in 20 mL of PBS or M9GC medium, in a 50 mL flask, inoculated with *P. aeruginosa* MPAO1 to a final $\text{OD}_{600} = 0.1$. Up to 10 implants were placed in a flask, which was incubated for 18 – 20 h at 37°C with 110 rpm shaking. The next day, each implant further incubated in M9GC or PBS with or without 1 $\mu\text{g/mL}$ of imipenem for up to 1 hour. The implants were washed once by briefly dipping into fresh PBS before treatment with 200 μM of DEACP in PBS or M9GC medium.

3.2.10.3 PAO1 *nirS::gfp* reporter assay

Overnight cultures of *P. aeruginosa* PAO1 *nirS::gfp* were diluted in fresh LB10 medium or PBS medium to a final OD_{600} of 0.2 and 0.4 respectively. Diluted samples were further incubated at 37°C with shaking at 200 rpm to $\text{OD}_{600} \sim 0.4$. Samples were then treated with imipenem to a final concentration of 0.2 $\mu\text{g/mL}$ and incubated under the same conditions for an hour prior to addition of 200 μM of DEACP and further incubation at 37°C with 200 rpm shaking for an hour. Subsequently, cells were spun down at $13\,000 \times g$ at 4°C for 5 min and washed twice with 2 mL PBS before resuspension in 500 μL of PBS. Two hundred μL of the resuspension was aliquoted into a well of a 96 well plate and GFP

fluorescence was quantified by a microplate reader with absorbance at 460 nm and emission measured at 510 nm.

3.2.10.4 Evaluation of MIC, MBC and MBEC of antibiotics on *P. aeruginosa*

MIC and MBC were evaluated as described in Section 3.2.3. Minimum biofilm eradication concentration (MBEC) was determined using a 96 well peg lid plate system. Overnight cultures of *P. aeruginosa* PAO1 were diluted 200 times in M9GC medium. Two hundred μ L of the diluted culture was added into each well of a 96 plate and biofilms were allowed to develop on the peg lids over the next 6 h with static incubation at 37°C. Subsequently, the biofilms were challenged with 200 μ L of antibiotics diluted in M9GC medium and ranging from (0 – 128 μ g/mL) for 2 h. The BacTitre Glo assay (G8231, Promega) was used to quantify cell activity following antibiotic treatment.

3.2.11 *In vivo* mouse intraperitoneal foreign-body infection (implant) model

This protocol has been approved by the NTU Institutional Animal Care and Use Committee under the approval number #A-0191.

3.2.11.1 Bacteria and implant preparation

Implants were prepared as described in section 3.2.10.2, with implants suspended overnight in 20 mL of *P. aeruginosa* MPAO1 inoculated in PBS and subsequently transferred to M9GC medium for induction of β -lactamase.

3.2.11.2 Infection and treatment of mice

BALB/c female mice, 7 – 8 weeks old, were anesthetized with Ketamine HCl (2 mL of 100 mg/mL stock) and Xylazine (0.5 mL of 20 mg/mL stock) in saline (7.5 mL, to a total volume of 10 mL). Ketamine-HCl was used at a final

concentration of ~ 2 mg/20 g body weight per mice while Xylazine was used at a concentration of 0.1 mg/20 g per body weight per mouse. The anaesthesia cocktail was injected intraperitoneally at a dose of 100 μ L per 20 g of body weight per mouse. Each mouse was determined to be completely anesthetized based on the absence of pain reflexes on the footpad. Following anaesthesia, the skin was sterilized with 70% ethanol before an incision wound was made. The implant is washed once in saline before it is placed in the peritoneal cavity. One hundred μ L of 200 μ M DEACP or S150 was then dripped into the implant before the wound was sutured and closed. The mice were then allowed to recover over the subsequent next 24 h.

3.2.11.3 Quantification of viable bacteria

At 24 h post infection (h.p.i.), each mouse was sacrificed by an overdose of ketamine-HCl, followed by cervical dislocation. The implant and spleen were extracted from each mouse, placed in 1 mL of saline and kept on ice. Biofilm from the implants were obtained via sonication at 37 Hz under degas mode for 5 min followed by sonication at 37 Hz under pulse mode for another 5 min. Bacteria present in the spleen were obtained through homogenization with a tissue grinder. The samples were then used for serial dilution and spot plating. Plates were incubated at 30°C overnight and colonies were counted the next day.

3.2.12 *In vivo* mouse cornea infection model

This protocol has been approved by the NTU Institutional Animal Care and Use Committee under the approval number #A-0229.

3.2.12.1 Bacteria

P. aeruginosa PAO1 strains were grown overnight in LB10 medium at 37°C with 200 rpm shaking. Overnight cultures were washed twice by centrifugation at 9

000 × g and the pellet was re-suspended in 0.9% NaCl (Saline) to a final OD₆₀₀ of 0.58 (McFarland standard no. 2) for use in infection.

3.2.12.2 Infection of mice

C57BL/6 female mice, 7 – 8 weeks old, were anesthetized as described (Section 3.2.11.2). Each cornea was then scarified using a small surgical blade by making parallel incisions, laying perpendicular to each other in a cross-hatch, over the corneal surface no deeper than the superficial stroma. Ten µL of the bacterial suspension, prepared as described above, was applied to each scarified cornea and the infection was allowed to develop over 24 h.

3.2.12.3 Treatment of mice

Each mouse was treated three times 24 h post infection (h.p.i), 28 h.p.i and 32 h.p.i. During treatment, mice were anesthetized using 2% inspired isoflurane for < 5 min while 5 µL of each treatment compound is applied to the infected scarified cornea. For experiments involving the cephalosporin-linked NO donor compound, DEACP, each treatment consisted of three compounds, 1 µg/mL IMIP dissolved in saline, 200 µM DEACP diluted in saline and 40 µg/mL colistin sulphate dissolved in saline, added sequentially at t = 0 min, t = 45 – 60 min and t = 60 – 75 min respectively. For experiments involving only antibiotic treatments, 4 – 400 µg/mL of the respective antibiotic was added at t = 0 min. Control groups consisted of infected mice treated with equivalent volumes of saline at similar time points.

3.2.12.4 Quantification of viable bacteria

At 48 h.p.i, each mouse was sacrificed by an overdose of ketamine-HCl, followed by cervical dislocation. The cornea was enucleated and suspended in 50 µL of saline in a 1.5 mL microfuge tube and kept on ice. Each cornea was homogenised

in two steps, first by grinding with a sterile micro-pestle (SIAL501ZZ0, Sigma Aldrich). Saline was then added to each tube a total volume of 500 μ L before the addition of sterile 3 mm glass beads (Z143928, Sigma Aldrich) to half the total volume of each tube. This was followed by 3 min of vortexing at moderate speed. To quantify viable bacteria, 100 μ L of the homogenate was added to 100 μ L of saline and serially diluted 1:10 in PBS. Ten μ L of each dilution was spot plated onto LB10 agar plates in triplicates and incubated at 37°C. Colonies were counted and expressed as CFU/mL.

3.3 Results

3.3.1 Summary and workflow of experiments

Several factors were evaluated and optimized for study of the compounds *in vivo*. Figure 3.2 and Table 3.3 provides a summary of the variables evaluated, as well as the corresponding results section.

Table 3.3 – Factors evaluated for their influence on NO release and dispersal

	System	Variable	Results	Section
<i>In vitro</i>	Cell-free	Kinetics of NO release	Batch-to-batch variation between current batch of compounds and previous studies (Yepuri <i>et al.</i> , 2013).	3.3.2
	Plate Biofilm	Type of inducer	NAG and NAM unable to induce biofilm dispersal. Imipenem the most effective in inducing biofilm dispersal.	3.3.4 – 3.3.5
		Concentration of IMIP used	Higher concentration of IMIP correlates to higher levels of β -lactamase induction, although higher levels of β -lactamase induction does not necessarily translate to a higher extent of dispersal.	3.3.5 – 3.3.7
		Duration of IMIP induction	≥ 45 min required for sufficient induction of β -lactamase activity for dispersal	3.3.7
		Dispersal activity	Differences observed between current batch of compounds and previously published results. As with the NO release kinetics of the compounds, this is likely due to batch-to-batch variation, as kinetics of NO release and compound dispersal activity was comparable.	3.3.6
		Comparison of dispersal	DEACP able to disperse both MPAO1 and PAO1 in a	3.3.10

		in MPAO1 and PAO1 strains	concentration dependent manner.	
		Medium of biofilm growth	No biofilm development in cells suspended in PBS.	3.3.11
		Effect of washing on extent of dispersal	Assessment of washing steps involved in mouse intraperitoneal implant model. Washing reduced the extent of dispersal observed.	
		Effect of multiple dosing	Multiple dosing of NO reduced the extent of dispersal across each dosing	3.3.15
	Implant	Medium of biofilm growth	No dispersal observed with biofilms grown in PBS as compared to biofilms developed in M9GC medium	3.3.11
	Planktonic	Medium and β -lactamase induction	No observable β -lactamase induction in cells grown in PBS as compared to cells grown in M9GC medium	3.3.12
		Medium of biofilm growth and NO detection	No detection of NO by PAO1 <i>nirS::gfp</i> reporter strain in cells resuspended in PBS as compared to cells grown in M9GC medium	3.3.12
<i>In vivo</i>	Implant	Medium of biofilm growth	High mortality observed in animals with implants biofilm grown in M9GC medium	3.3.13
		Treatment	Unexpectedly higher CFU counts from both implants and spleen samples of NO-donor treated mice	
	Keratitis	Treatment	Variation in results across independent studies	3.3.16

Figure 3.3 – Summary of workflow of current study. Concentrations and conditions used were optimized using *in vitro* models before testing in animal models.

3.3.2 NO release kinetics of cephalosporin-linked NO donor prodrugs

To ensure consistency between batches of synthesized cephalosporin-linked NO donor prodrugs, NO release from the cephalosporin were evaluated using an NO analyzer. Exponential release of NO from the pro-drugs was observed upon addition of penicillinase. Kinetics of NO release from the activated prodrug corresponded to the half-life of the NONOate components (Figure 3.4, Table 3.4). NO release from UW-2, which contains a proli-NONOate component ($t_{1/2} = 1.8$ s at 37°C, pH = 7.0), occurred over a shorter duration ($t = 4 - 7$ min) with a faster

rate of NO release at 691.9 ± 186.8 nM/ min. In contrast, the maximum NO release for UW-0 (DEACP), 1 and 4 – 6, which consist of a DEA NONOate with a half-life of 2 min under physiological conditions, occurred around 5 – 11 min and with NO release rates of 162.4 – 187.8 nM/min. UW-3 and UW-5 contain Pen-pip and Pyrim-pip NONOates, respectively, with similar half-lives of 2 min, and had maximum rates of NO release of around 150 ± 20.78 nM/min and 235.5 ± 29.54 , respectively.

Table 3.4 – Estimated rate of NO release by each compound upon the addition of penicillinase.

Compound	Rate of NO release (nM min ⁻¹)*
UW-0	187.8 ± 10.19
UW-1	178.6 ± 11.11
UW-2	691.9 ± 186.8
UW-3	150 ± 20.78
UW-4	181.3 ± 16.2
UW-5	235.5 ± 29.54
UW-6	162.4 ± 12.07

*Rates of NO release are expressed as nM min⁻¹ $\pm$ S.E and were determined via linear regression of data points lying between t = 7 – 10 min for UW- 0 – 1 and UW-3 – 6 and between t = 4 – 6 min for UW-2.

Figure 3.4 – NO release kinetics of cephalosporin-based NO donor prodrugs. NO release kinetics of prodrugs with a DEACP NONOate component linked to different cephalosporin backbones (a) or other NONOates components associated with the cephalosporin, cefaloram (b) as measured by a free-radical NO analyser.

3.3.3 – MIC of cephalosporin-linked NO donor compounds

While cell-free NO release from UW-0 to UW-6 had been previously evaluated in Yepuri *et al.* (2013), the MIC of the compounds had not been tested. Evaluation of the MIC would allow the selection of a suitable working concentration of hybrid compound. At present, using a non-toxic concentration of the hybrid drug is desired to distinguish the effects of NO release from killing activity. Furthermore, hybrid compounds are known to display modulated activities in one or more of their respective single constituents (Kutty *et al.* 2013, Tran *et al.* 2013). As such, the MIC of the hybrid compounds were evaluated and compared to the activity of the antibiotic alone.

All hybrid compounds display similar high MICs as that of the cephalosporin backbone (Table 3.5). This concentration corresponds to approximately > 230 – 280 μ M of the hybrid compound, indicating that they are relatively non-toxic at the concentrations to be used for inducing dispersal.

Table 3.5 – MIC of cephalosporin backbone and hybrid compounds.

Antibiotics/ Compound	MIC (μ g/mL)*	MBC (μ g/mL)*
Cefaloram	>128	>128
UW-0	>128	>128
UW-1	>128	>128
UW-2	>128	>128
UW-3	>128	>128
UW-4	>128	>128
UW-5	>128	>128
UW-6	>128	>128

3.3.4 MIC of potential inducers of β -lactamase in *P. aeruginosa*

The bactericidal properties of imipenem have implication on its use as a β -lactamase inducer in our studies and in future applications where dose patterns of antibiotics may influence the selection of resistance and bacterial eradication

(Opatowski *et al.* 2010). Hence, other potential inducers of β -lactamase production, namely NAG and NAM, were evaluated. These two monosaccharides were selected as induction of β -lactamase in *P. aeruginosa* via the AmpG-AmpR-AmpC pathway is promoted in the presence of GlcNAc-anhydro-MurNAc-peptides and 1,6-anhydroMurNAc-oligopeptides (Zeng and Lin 2013). It is hence of interested to assess if partial components of these peptides, such as NAG and NAM, which are commercially available, may also induce β -lactamase production. Ceftazidime, a commonly used cephalosporin against *Pseudomonas spp.*, but is not known to induce β -lactamase production, was included as a negative control. In addition, the lead compound DEACP (UW-0), which contains a cephalosporin component of an undefined class, was evaluated for β -lactamase production.

To determine a suitable concentration for the use of these compounds for β -lactamase induction, their minimum Inhibitory concentration (MIC), which is the lowest drug concentration that prevents visible microorganism growth after overnight incubation, were evaluated. Although the MIC data of *P. aeruginosa* for commercial antibiotics such as imipenem and ceftazidime are available, actual MIC can show a large variation due to strain-to-strain differences and assay variation due to differences in testing conditions (Luyt *et al.* 2014, Mouton *et al.* 2017). The highest MIC for non-resistant isolates or “wildtype” strains is known as the epidemiological cut-off value (ECOFF). For *Pseudomonas spp.*, infections are classified as susceptible, intermediate or resistant to imipenem based on whether their MICs fall below 4 mg/L or above 8mg/L. In the work presented here, the MIC of imipenem for the lab strain PAO1 was 1 μ g/mL while for the clinical strain *P. aeruginosa* D25 it was 16 μ g/mL. The MICs of the ceftazidime was at 8 μ g/mL for the PAO1 strain. Both NAG and NAM were not toxic to *P. aeruginosa* PAO1, enabling growth even when used up to 512 μ g/mL.

Table 3.6 – Minimum inhibitory and bactericidal concentrations of compounds used in the evaluation of induction of β -lactamase activity.

Antibiotics/ Compound	MIC ($\mu\text{g/mL}$)*	MBC ($\mu\text{g/mL}$)*
Imipenem	1	4
Ceftazidime (Caz)	8	16
N-acetylglucosamine (NAG)	> 512	> 512
N-acetylmuramic acid (NAM)	> 512	> 512

*MICs and MBCs are the mode of three biological replicates.

3.3.5 Activation of β -lactamase by DEACP and inducers of β -lactam production

Previous studies involving cephalosporin-linked NO donor prodrugs made use of an arbitrary sub-inhibitory concentration of imipenem ranging from 0.3 – 0.5 $\mu\text{g/mL}$ for induction of β -lactamase. Other studies had also shown that the highest level of β -lactamase production in *P. aeruginosa* is induced in the presence of 0.25 – 2 $\mu\text{g/mL}$ of imipenem (Giwerzman *et al.* 1991). Unlike in our experiments, the study was carried out over the course of 16 h. It is not known if slight changes in imipenem concentrations used within the 0.25 – 2 $\mu\text{g/mL}$ range would affect the extent of β -lactamase induction in *P. aeruginosa*. In addition, we would like to evaluate NAG, NAM and the lead compound, DEACP (UW-0) for the ability to induce β -lactamase production. We hypothesize that a higher extent of β -lactamase induction would better promote hybrid drug cleavage and release of NO. Induction of β -lactamase was carried out under similar conditions used for subsequent assays.

Cultures pre-treated with the compounds were incubated with nitrocefin, a chromogenic β -lactamase substrate that changes from yellow to red upon hydrolysis by the enzyme. Sub-MIC and MIC concentrations of antibiotics (imipenem and ceftazidime) were used for induction of β -lactamase. For NAM and NAG, concentrations between 10 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ were selected for testing. DEACP, the NO donor prodrug consisting of a cephalosporin backbone,

was also evaluated at concentrations between 100 – 500 μM . A control treated under the same conditions with dH_2O was included.

Figure 3.5 – β -lactamase activity of samples treated with different compounds. β -lactamase activity of samples treated for 1 h with 0.5 – 2 $\mu\text{g/mL}$ of imipenem (a), 4 – 8 $\mu\text{g/mL}$ of ceftazidime (b), 10 or 100 $\mu\text{g/mL}$ of NAG or NAM (c) and 100 – 500 μM of DEACP (d) over 30 min. Experiments were repeated twice independently and data points plotted are based on one representative data set. β -lactamase activity, represented as the rate of hydrolysis of nitrocefin over time, following 1 h induction with 0.5 – 2 $\mu\text{g/mL}$ of imipenem was also determined (e). Data was based on two independent experiments and error bar represent the standard deviation of the mean – *, $p \leq 0.05$.

Of the compounds tested, only samples treated with imipenem displayed β -lactamase activity, indicated by an increase in absorbance at 490 nm over time (Figure 3.5 a–d). Induction of β -lactamase activity upon treatment with imipenem was dependent in part on the concentration of the antibiotic used (Figure 3.5 e), as 0.5 $\mu\text{g/mL}$ of imipenem resulted in a significantly lower β -lactamase activity ($p < 0.05$) of $3.33 \pm 0.92 \text{ nmol min}^{-1} \text{ mL}^{-1}$ as compared to 1–2 $\mu\text{g/mL}$ of imipenem, which resulted in a β -lactamase activity of 7.26 ± 0.71 and $7.49 \pm 0.99 \text{ nmol min}^{-1} \text{ mL}^{-1}$, respectively.

3.3.6 Dispersal activity of cephalosporin-based NO donor compounds

Dispersal activity of the cephalosporin-linked NO donor compounds had been previously evaluated (Yepuri *et al.*, 2013). However, NO activity may differ slightly depending on the growth conditions (Appendix A). In addition, NO release from the compounds had also differ slightly from previously published results, in which UW-4 and UW-6 displayed a slightly higher rate of NO release than UW-1 and UW-0, despite all compounds having the same NONOate component, following the addition of β -lactamase. As such, dispersal activity was re-evaluated.

All cephalosporin-based NO donor compounds induced dispersal at concentrations between 100 – 500 μM in a concentration dependent manner (Figure 3.6). Dispersal was correlated with an increase in absorbance at OD₆₀₀ (Figure 3.6c). DEACP, UW-1, 4 and 6, which have the same NONOate component, DEA NONOate, induced dispersal at 200 μM and 500 μM , ranging from 42 – 55% and 55 – 61% reduction of biomass, respectively. DEACP, UW-1 and 4 showed similar dispersal between 39.6 – 49.8%, at 100 μM . However, UW-6 displayed a lower extent of dispersal (10%) at 100 μM .

UW-2, which consists of a proli NONOate with a half-life of 2 s under the conditions tested, dispersed 39.8%, 45.4% and 44.3% of biofilms at 100, 200 and

500 μ M, respectively. Amongst the compounds tested, UW-3 had the lowest extent of dispersal at all concentrations tested, resulting in 0.98%, 19.9% and 43.1% reduction in biofilm at 100, 200 and 500 μ M, respectively.

Figure 3.6 – Effect of treatment with NO donor prodrugs on dispersal, CV staining and planktonic OD₆₀₀. Activity of cephalosporin-based NO donor prodrugs UW-0 to UW-6, against *P. aeruginosa* biofilms as expressed in the percentage biomass changes with respect to the control group (a), as well as OD₅₅₀ (b) and OD₆₀₀ (c) changes upon addition of UW0-UW6 following pre-treatment of 1 μ g/mL of imipenem. Representative photo of CV staining following treatment with different compounds (d). Control group refers to samples treated with 1 μ g/mL of imipenem at $t = 5$ h. Bars represent data from three biological replicates while error bars represents the standard deviation from the mean – *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

3.3.7 Factors influencing dispersal by DEACP

While 0.5 – 2 $\mu\text{g/mL}$ of imipenem used was the most effective at inducing β -lactamase production, it is not known if improvements in β -lactamase production translates to improvements in dispersal, which is dependent on active live cells, given the bactericidal properties of the antibiotic. This factor was also not evaluated in previous studies, which made use of arbitrary concentrations of imipenem below its MIC for β -lactamase induction. As such, the effect of addition of different concentrations of imipenem on dispersal was evaluated.

Figure 3.7 – Effect of absence of imipenem on dispersal by DEACP. Dispersal activity of 100 – 500 μM of DEACP in the absence of imipenem pre-treatment (a), and corresponding changes in crystal violet staining (b, c) or planktonic optical density (d). Bars represent data from two biological replicates and error bars represent the standard deviation of the mean.

Treatment with only DEACP, without prior addition of imipenem, required for β -lactamase induction, resulted in a lack of biofilm dispersal (Figure 3.7). Although the MIC of imipenem was approximately 1 $\mu\text{g/mL}$ (Table 3.4), a range of imipenem concentrations were found to be suitable for use in biofilm dispersal (Figure 3.8). Increasing the imipenem concentrations used correlated with the extent of β -lactamase induction (Figure 3.5 a, e), but was inversely proportional to the extent of dispersal (Figure 3.8). The use of 0.5 $\mu\text{g/mL}$ of imipenem resulted in $\sim 60\%$ reduction in biofilm following treatment with 200 μM of DEACP ($p < 0.001$), while 1 and 2 $\mu\text{g/mL}$ of imipenem gave approximately 44% and 36% reduction in biofilm ($p < 0.01$).

Figure 3.8 – Effect of imipenem concentration on dispersal by DEACP. Effect of the concentration of imipenem pre-treatment on dispersal activity by 200 μM of DEACP (a), corresponding changes in crystal violet staining quantified (b) and representative photograph of crystal violet staining patterns (c). Bars represent data from two biological replicates and error bars represent the standard deviation of the mean as compared to controls without DEACP treatment - *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Confocal studies (Figure 3.9, 3.10) indicated that both 0.5 – 1 $\mu\text{g/mL}$ of imipenem could induce similar extents of dispersal as the spontaneous NO donor S150 (~ 26% reduction in biovolume), resulting in approximately ~ 31% and ~ 28% reduction in biovolume, respectively. similar extents of dispersal as the spontaneous NO donor S150 (~ 26% reduction in biovolume), resulting in approximately ~ 31% and ~ 28% reduction in biovolume, respectively. There was no significant difference in the percentage of dead cells when treated with 0.5% or 1% imipenem. (Figure 3.9). Additionally, similar or higher CFU were recovered from planktonic fractions of samples treated with 1 $\mu\text{g/mL}$ of imipenem as compared to samples treated with 0.5 $\mu\text{g/mL}$ of imipenem, while samples treated with 2 $\mu\text{g/mL}$ of imipenem showed a 23 – 37 % reduction in CFU in comparison to 0.5 $\mu\text{g/mL}$ of imipenem treated sample (data not shown).

Figure 3.9 – Biovolume of samples treated with NO donor compounds. Biovolume of samples treated with 200 μM of the spontaneous NO donor S150 or the pro-drug DEACP with pre-treatment of 0.5 – 1 $\mu\text{g/mL}$ of imipenem (a) and composition of live/dead cells within each biofilm (b). Bars represent data from three image stacks and error bars represent the standard deviation of the mean - *, $p \leq 0.05$; **, $p \leq 0.01$.

Figure 3.10 – Confocal images of PAO1-gfp biofilms treated with NO donor compounds. Representative confocal images of samples treated with 200 μM of a spontaneous NO donor, S150 (b) or 200 μM of DEACP (d, f), and their respective controls (a, c, e).

1 µg/mL of imipenem was selected for use in further studies despite showing a lower extent of dispersal than the use of sub-inhibitory concentration of imipenem. This was based off similar cell death with use of 1 µg/mL and 0.5 µg/mL of imipenem and higher β-lactamase production with 1 µg/mL of the antibiotic, together with expectations that *in vivo* biofilms would be more resistant to such treatments than *in vitro* biofilms (van Ogtrop *et al.* 1990).

As gene induction is time-dependent, the effects of the duration between imipenem addition and DEACP (UW-0) treatment was further evaluated (Figure 3.11). Induction of β-lactamase expression was rapid and the addition of imipenem 60 min prior to the addition of DEACP was sufficient for dispersal of the biofilm under the conditions tested ($p < 0.01$) This was consistent with previous studies in which biofilms were either grown with β-lactamase or added 1 h prior to the addition of the hybrid compounds (Kutty *et al.* 2013, Yepuri *et al.* 2013).

It was of interest to determine the minimum time required for dispersal activity to be observed following β-lactamase induction so that an animal's time under anaesthesia could be shortened accordingly during animal studies. Co-administration of imipenem and DEACP at the same time at $t = 6$ h did not result in any substantial dispersal with 30 min of treatment with DEACP. Evaluation of β-lactamase activity indicated that approximately 45 min was required for the onset of observable β-lactamase activity in the bacterial sample following treatment with 1 µg/mL imipenem. Correspondingly, samples treated with imipenem 45 – 60 min prior to assessment of β-lactamase activity hydrolyse nitrocefin immediately, while samples treated with imipenem 15 – 30 min prior require an additional 30 to 15 min respectively before an exponential increase in β-lactamase activity was observed.

Figure 3.11 – Effect of duration of imipenem treatment on β -lactamase induction and biofilm dispersal. Effect of the duration of 1 $\mu\text{g/mL}$ imipenem treatment (0 – 60 min prior to addition of DEACP) on the extent of biofilm dispersal by 200 μM DEACP (a – b), with corresponding increases in planktonic optical density following dispersal (c). Bars represent data from two biological replicates and error bars represent the standard deviation of the mean - **, $p \leq 0.01$. Photograph of CV staining pattern following DEACP treatment (d). Duration leading to the onset of observable β -lactamase activity following treatment with 1 $\mu\text{g/mL}$ imipenem 0 – 60 min prior to carrying out the assay (e), with one representative data set plotted.

3.3.8 Cytotoxicity of DEACP

As DEACP was to be further evaluated for its use in animal models, its cytotoxicity was first assessed using H1299 lung epithelial cells via detection of lactate dehydrogenase (LDH), which is a cytosolic enzyme released into the medium by dead or dying cells (Figure 3.12). The LDH assay was selected as it is suited for the measurement of acute toxicity, which corresponds to the short duration of exposure to the compounds in *in vitro* and *in vivo* experiments. This contrasts with metabolic assays such as the MTT assay which are better suited for detection of later stages of cytotoxicity and cell death (24 – 96 h), when metabolic activity of the cell is severely reduced (Sumantran 2011).

As different cell types have different levels of basal LDH activity, a preliminary experiment was first carried out to ensure that the LDH signal falls within a linear signal range for the cell number used. The LDH signal appeared to be linear when tested in the range of 0 – 160,000 cells, with $R^2 = 0.9661$ (Figure 3.12a). A cell number of 100,000 cells per well was subsequently chosen for further evaluation of compound cytotoxicity. DEACP did not appear to be cytotoxic when used at 100 – 1000 μM under the conditions tested (<5% cytotoxicity) (Figure 3.12b)

Figure 3.12 – Measurement of LDH signal and cytotoxicity of DEACP in H1299 cells. LDH signal from 0 – 160 000 of H1299 cells following complete lysis (a) and cytotoxicity of 100 – 1000 μM of DEACP and equal volume (0.1 – 1%) of DMSO (b). Each point or bar represents data from three replicates and error bars represent the standard deviation of the mean.

3.3.9 Selection of mouse infection models

Following assessment of conditions that may affect NO release from the compounds, an infection model was selected for further evaluation of the activity of the compound *in vitro* and *in vivo*.

The mouse intraperitoneal foreign-body infection model was first selected based on multiple reasons. Firstly, *P. aeruginosa* is frequently associated with chronic infections on medically implanted foreign devices (Braxton *et al.* 2005, Neut *et al.* 2005). These infections are generally not cleared until device removal (Neut *et al.* 2005). Subsequently, re-implanted devices are prone to recolonization by similar bacteria (Cherney and Amstutz 1983). As such, more methods could be developed for the treatment of such medically device associated infections.

Secondly, unlike *P. aeruginosa* associated lung infections, when NO may be delivered directly to the infection site through inhalation of NO gas (Howlin *et al.* 2017, Miller *et al.* 2013), delivery of NO to other infection sites, such as an implant, poses more challenge due to the reactivity of NO following its release from NO-donors. Using the intraperitoneal foreign body infection model hence enables one to assess if targeted delivery of NO may be a viable option for treatment of infections where direct use of NO gas is not a suitable choice.

Lastly, the model had been previously used for proof-of-concept studies to show that dispersal is a viable treatment strategy against biofilm-based infections (Christensen *et al.* 2013). In that study, induction of a plasmid encoded phosphodiesterase reduced cdGMP levels, leading to dispersal. Such an increase in PDE activity and lowering of cdGMP levels were also associated with dispersal by NO (Barraud *et al.* 2009a). Hence, this suggests that the model is suitable for dispersal studies and bacteria clearance *in vivo*. The results and limitations of the intraperitoneal foreign-body infection model would be presented and discussed in later sections (Section 3.3.13, 3.4).

Subsequently, a mouse keratitis (cornea infection) model was used for evaluation of the compounds. Like the intraperitoneal foreign body infection model, the keratitis model provides an infection situation in which targeted NO delivery is a more viable option than the use of NO gas. The formation of *P. aeruginosa* biofilms in this model had been previously characterized, and establishment of the biofilm had been observed to be similar to *P. aeruginosa* infections in cystic fibrosis lungs (Yam *et al.* 2017). Biofilm formation was cdGMP dependent, and at initial stages, appear to be due to downregulation of PDEs, such as *nbdA*, *dipA*, *mucR* and etc, with the former three genes known to be involved in NO-induced dispersal (Li *et al.* 2013a, Roy *et al.* 2012). Furthermore, the use of a low cdGMP expressing *P. aeruginosa* strain, typically associated with a planktonic or dispersed state of cells, was also found to promote phagocyte penetration and bacterial cell clearance, suggesting that any dispersal by the NO-donor prodrug may also aid in biofilm eradication (Yam *et al.* 2017).”

3.3.10 – 3.3.13 The effect of NO prodrugs in alleviating infection in a mouse foreign-body infection model

Prior to *in vivo* intraperitoneal experiments, dispersal by DEACP was further evaluated *in vitro* using the MPAO1 strain, which was less virulent at a similar dose and would later be used in the mouse foreign-body infection model. A switch of strains for the intraperitoneal foreign body model was due to animal death in previous trial runs involving the lab PAO1 strain. The reasons accounting for the deaths would be further discussed in later sections (section 3.4). Both *P. aeruginosa* PAO1 and MPAO1 behaved similarly, displaying increased dispersal, with a decrease in CV staining and an increase in planktonic optical density, over a range of DEACP concentrations of 10 – 200 μ M (Figure 3.13). At 200 μ M, DEACP dispersed approximately 45% and 34% of *P. aeruginosa* PAO1 and MPAO1 biofilms, respectively ($p < 0.0001$).

3.3.10 Comparison of dispersal in *P. aeruginosa* PAO1 and MPAO1 strains

Figure 3.13 – Dispersal by DEACP in *P. aeruginosa* PAO1 and MPAO1. Dispersal activity of 10 – 200 μ M of DEACP following pre-treatment with 1 μ g/mL imipenem (a), and corresponding changes in crystal violet staining (b, c) or planktonic optical density (d). Bars represent data from two biological replicates and error bars represent the standard deviation of the mean – *, p $\leq$ 0.05; ***, p $\leq$ 0.001; ****, p $\leq$ 0.0001.

3.3.11 Assessment of dispersal in implants

In addition to the use of *P. aeruginosa* MPAO1 instead of the PAO1 strain, several other changes were made to optimise conditions for compound use in *in vivo* mouse foreign-body infection models as compared to *in vitro* plate models. In *in vivo* mouse models, *P. aeruginosa* biofilms were formed on silicone

implants suspended in PBS overnight while in *in vitro* plate models, *P. aeruginosa* biofilms were formed on polystyrene plates over the course of 6 h.

To evaluate biofilm development and dispersal in PBS as compared to M9GC medium, MPAO1 was inoculated in both M9GC medium ($OD_{600} \sim 0.05$) and PBS ($OD_{600} \sim 0.05 - 0.5$) and the biofilm was allowed to develop using the plate assay as described (Section 3.2.4). When inoculated in PBS, biofilms neither formed well nor did they reach sufficient biomass for effective quantification by CV staining (Figure 3.14 a, b).

Figure 3.14 – Effect of NO treatment on MPAO1 biofilms developed in PBS or M9 medium. Biofilm biomass of MPAO1 inoculated in M9GC medium ($OD_{600} \sim 0.05$) or PBS at different initial inoculum ($OD_{600} \sim 0.05 - 0.5$) 6 h post incubation (a) and photograph of CV stain (b). CFU changes of silicone implants, incubated in M9GC medium or PBS inoculated with MPAO1 to a final $OD_{600} \sim 0.1$, upon treatment with 200 μ M of S150, 1 μ g/mL imipenem (IMIP), or imipenem with 200 μ M of DEACP (c) and the percentage change in CFU upon treatment with S150 or imipenem with DEACP as compared to their respective controls, samples treated with buffer or imipenem only (b). For (a), bars represent data from two biological replicates and error bars represent the standard deviation of the mean. For (c) and (d), each data point represents CFU from one biological replicate and error bars represent the standard error of the mean - ** $p \leq 0.01$.

Biofilms were then developed on silicone implants in M9GC medium or PBS inoculated with MPAO1 ($OD_{600} \sim 0.1$), before each implant was transferred to 1 mL of the respective medium or buffer and treated with imipenem, imipenem plus DEACP or S150. Enumeration of CFU indicated that biofilms developed in PBS were not responsive to dispersal by 200 μ M of S150 or a combination treatment of 1 μ g/mL imipenem and 200 μ M DEACP as compared to biofilms developed in M9GC medium (Figure 3.14c, d). Biofilms developed and treated in M9GC medium dispersed by approximate 33 and 35% when treated with S150 or imipenem and DEACP respectively, as compared to controls treated with PBS or imipenem only ($p < 0.01$).

3.3.12 β -lactamase induction and NO release in PBS

To evaluate the NO release of DEACP in *P. aeruginosa* suspended in PBS, a PAO1 *nirS::gfp* strain, which has a green fluorescence protein (GFP) reporter gene fused to the *nirS* promoter, was used (Barraud *et al.* 2006). The presence of NO activates transcription from the *nirS* promoter, leading to an increase in GFP signal.

Exposure of cells to imipenem and DEACP in LB medium gave ~3 times higher fluorescence ($p \leq 0.0001$) in comparison with cells exposed to only PBS or imipenem (Figure 3.14 a). Exposure of PBS resuspended cells to imipenem and DEACP did not result in significant increases in Gfp expression. It was hypothesized that decreased dispersal or reduced NO release from DEACP post imipenem treatment was due to lowered activity of the cells when resuspended in PBS due to carbon starvation. Measurement of ATP levels in *P. aeruginosa* MPAO1 grown in M9GC medium for 6 h or resuspended in PBS for the same amount of time indicated that ATP levels were comparable between the two groups, after adjustments for differences in OD_{600} . Cells suspended in PBS displayed a slightly higher luminescence signal, correlated with higher amounts of ATP, of $\sim 2.2 \times 10^6$ RLU/ OD_{600} as compared to $\sim 1.9 \times 10^6$ RLU/ OD_{600} in M9GC medium (Figure 3.15 b). Quantification of β -lactamase activity in cells

grown under the same conditions indicated that β -lactamase activity was present when cells grown in M9GC medium were treated with 1 $\mu\text{g/mL}$ of imipenem. However, cells resuspended in PBS did not show any β -lactamase activity, even when treated with a higher concentration of 2 $\mu\text{g/mL}$ of imipenem (Figure 3.15 c, d).

Figure 3.15 – IMIP and DEACP treatment on NO detection and β -lactamase induction in *P. aeruginosa*. Effect of growth of MPAO1 in rich or minimum medium as compared to resuspension in buffer. Signal from the PAO1 *nirS::gfp* reporter strain upon treatment with 0.2 $\mu\text{g/mL}$ imipenem in the presence or absence of 200 μM of DEACP in rich LB medium or PBS buffer (a) and relative amounts of ATP present in MPAO1 cells grown in M9 medium as compared to cells resuspended in PBS, adjusted for differences in OD₆₀₀ (b). Bars represent data from two biological replicates error bars represent the standard deviation of the mean – ****, $p \leq 0.0001$. Representative data of the extent of β -lactamase induction in the presence of 0 – 2 $\mu\text{g/mL}$ of imipenem in M9 medium (c) or PBS (d).

3.3.13 *In vivo* mouse intraperitoneal foreign body infection model

Following assessment of dispersal from implants using different strains and growth conditions, the experiment was carried out in mice with *P. aeruginosa* MPAO1 biofilms developed on implants in M9GC medium. Initial experiments resulted in high mortality of mice, with eleven of fifteen mice dead within 24 h post-surgery. Five of five mice of the imipenem and DEACP treated group, four of five mice of the imipenem treated group and two of five mice of the PBS treated control group accounted for the total deaths.

Figure 3.16 – Results of mouse intraperitoneal foreign body infection model. CFUs obtained from implants prior to surgery and implants and spleens collected from infected mice treated with saline (control), 200 μ M S150 (S150), 1 μ g/mL imipenem (IMIP) or 1 μ g/mL imipenem with 200 μ M DEACP (IMIP + DEACP) (a). Each data point represents the CFU/mL from the treatment of one cornea while error bars represent the standard error of the mean Photos of mice from control group (b) or NO donor compound treated group (c).

Further experiments were then carried out using MPAO1 biofilms developed on implants in PBS. No deaths were recorded within 24 h post-surgery. Before implantation, both control groups (treated with PBS) and imipenem treated groups displayed similar CFU counts (Figure 3.16 a). Following exposure to 200 μ M DEACP or S150, or equal volumes of PBS over 24 h, the mice were sacrificed and both implants and spleen collected for CFU enumeration. Higher CFUs were recorded in both implant and spleen samples treated with S150 and DEACP as compared to their respective controls (Figure 3.16 b, c). Mice treated with S150 displayed a 0.57 $\log_{10}$ fold higher MPAO1 CFU counts in extracted implants and 3.29 $\log_{10}$ fold higher CFU in excised spleen as compared to PBS treated mice. Similarly, mice treated with DEACP had 2.15 $\log_{10}$ fold higher CFU in implants and 2.69 $\log_{10}$ fold higher CFU in excised spleen.

3.3.14 - 3.3.16 NO mediated control of infections in a mouse keratitis (cornea infection) model

3.3.14 Evaluation of MIC and MBEC of colistin

Inherent to the mouse intraperitoneal foreign-body infection model are systemic infections and higher mortality rates. Therefore, the compounds were evaluated, instead, using the cornea infection model. In this model, the infection is localized to the cornea and unlikely to lead to mortality prior to the animals being sacrificed for CFU enumeration. The model also enables straightforward application of the treatments.

An additional treatment step with antibiotics was considered for the mouse keratitis model. This was because although *P. aeruginosa* with lower cdGMP levels, i.e. corresponding to a more dispersed lifestyle, form smaller aggregates with increased phagocytic cell penetration, in general, *P. aeruginosa* biofilms still display high tolerance towards host phagocytic cells (Yam *et al.* 2017). Colistin, a commonly administered antibiotic against *P. aeruginosa* infections

that has been indicated for use in *P. aeruginosa* related ocular infections, was selected. The use of an antibiotic treatment necessitates that a sufficiently high concentration of the antibiotic is used for observation of a clearing effect, but low enough to ensure that clearance of the biofilm is due to a synergistic combination effect with the NO-donor compound, and not the antibiotic alone. For this purpose, the MIC and MBEC of colistin was evaluated and found to be at 1 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$ respectively.

Figure 3.17 – MBEC of colistin. Biofilm formation in M9GC medium on peg plates over time (a) and survival rates of PAO1 biofilms challenged with 1 – 512 $\mu\text{g/mL}$ of colistin. Each data point represents data from two biological replicates and error bars represent the standard deviation of the mean.

For evaluation of MBEC, the biofilm was first developed on peg plates before being challenged with antibiotics. Biofilm development on the peg plates in M9GC medium was evaluated and the biofilm was found to naturally disperse after $t = 8$ h (Figure 3.17 a). The plate was hence challenged with 1 – 512 $\mu\text{g/mL}$ of colistin in fresh M9GC medium at $t = 6$ h, prior to biofilm dispersal for 2 h (Figure 3.17 b). Colistin was found to reduce survival in a concentration dependent manner when administered at 2 – 512 $\mu\text{g/mL}$. At 4 $\mu\text{g/mL}$, colistin reduced the survival of cells within the biofilm by 44%, while administration of 8 $\mu\text{g/mL}$ colistin reduced survival by 69%.

3.3.15 Effects of multiple NO doses on biofilm dispersal

In the mouse keratitis model, treatment was carried out three times over the course of a day. Such multiple dosing was necessary as the avascularity of cornea, together with poor drug penetration in the stroma necessitates frequent local antibiotic administration (Gokhale 2008). The plate model was used to evaluate the effect of such multiple doses of NO treatment on biofilm dispersal. In the mouse keratitis model, three treatments of imipenem, DEACP and/or colistin were administered at intervals of 4 h. However, in the plate model, biofilms were found to disperse by 8 – 9 h due to carbon starvation. Hence, treatments were administered at intervals of 2 h over 6 h. Furthermore, at least 45 min was required for induction of β -lactamase to a suitable level for DEACP activation. Following which, a further 15 – 30 min of exposure to DEACP is required for dispersal before treatment with colistin. As such, the treatments with imipenem, DEACP and colistin were not suitable for the intervals used in the plate model.

Instead, a spontaneous NO donor, S150, was used. S150, at 200 μ M could induce dispersal within 15 – 30 min of treatment, to the same extent as the combination therapy of imipenem and DEACP (Figure 3.9, 10). However, 20 μ M of S150 does not induce any significant amounts of dispersal when used under the same conditions (data not shown). In the wildtype strain, MPAO1, pre-treatment with both high (200 μ M) or low (20 μ M) concentrations of S150 reduced the extent of dispersal by 200 μ M of S150 at $t = 6$ h (Figure 3.18a, b). A single dose of S150 at $t = 6$ (treatment 1) induced dispersal of ~ 52%, while pre-treatment with high concentrations of S150 (treatment 2) reduced the extent of dispersal, as compared to the control, to ~25%. Pre-treatment with low concentration of S150 (treatment 3) further reduced the dispersal to ~13%. The reduction in extent of dispersal with either treatment 2 or 3 was significant ($p \leq 0.05$) when compared to the dispersal by treatment 1 (Figure 3.18 b).

Figure 3.18 – Effect of multiple NO dosing on dispersal in *P. aeruginosa* WT and *fhp* transposon mutant strain. Effect of multiple NO dosing on biomass (a, c) and percentage biofilm change (b, d) of WT MPAO1 strain (a, b) or MPAO1 *fhpF06::ISlacZ/hah* strain (c, d). Samples were untreated (control), treated once at t = 6 h with 200 μ M of S150 (1), treated thrice with 200 μ M of S150 at t = 2, 4, and 6 d (2) or thrice with 20 μ M of S150 at t = 2 and 4 h and 200 μ M of S150 at t = 6 h (3). Bars represent data from three biological replicates and error bars represent the standard deviation of the mean – *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Unlike MPAO1, MPAO1 *fhp-F06::ISlacZ/hah* that has *fhp* inactivated, did not show any reduction of in dispersal when pre-treated with both high and low concentrations of S150 (Figure 3.18 c, d). The extent of dispersal in the strain was dependent on the extent of exposure to S150, with a treatment 1 resulting in ~69% dispersal, while treatment 3 reduced CV staining by ~75%. Treatment 3, with multiple high doses of S150 further increases the extent of dispersal to ~81%.

The increases in the extent of dispersal in the transposon mutant strain in both treatment 2 and 3 were significant ($p \leq 0.01$) as compared dispersal by treatment 1.

3.3.16 Mouse keratitis infection

Following assessment of MIC and MBEC of colistin on PAO1. The effect of antibiotic treatment alone was evaluated using the mouse keratitis infection model. Based on the MBEC result, where 4 $\mu\text{g/mL}$ of colistin resulted in a large decrease in survivability of cells within the biofilm, the effect of 4 – 400 $\mu\text{g/mL}$ of the antibiotic on CFU was evaluated. In the keratitis infection model, 4 – 40 $\mu\text{g/mL}$ of colistin did not have any significant effect on the CFUs as compared to the control group treated with saline, while the use of 400 $\mu\text{g/mL}$ of colistin reduced CFUs by approximately 1.9 Log_{10} fold.

It was hypothesised that the use of a sub-lethal concentration of colistin would synergistically reduce biofilms present on the cornea following dispersal by DEACP. Hence, a concentration of 40 $\mu\text{g/mL}$ colistin was selected in subsequent keratitis infection studies. Three independent studies were subsequently carried out using previously determined concentrations of 1 $\mu\text{g/mL}$ imipenem, 200 μM DEACP and 40 $\mu\text{g/mL}$ colistin. Overall, a trend could be observed in that a combination treatment of imipenem, DEACP and colistin generally leads to a lower CFU count in comparison to groups treated with only one (imipenem) or two compounds (imipenem with colistin or DEACP) (Figure 3.19b). However, the differences were not statistically significant. In relation to the saline treated control group, treatment with imipenem reduced the CFU by 0.24 Log_{10} fold, treatment with imipenem and DEACP or colistin reduced the CFU by 0.41 or 0.69 Log_{10} fold respectively while the combination treatment of imipenem, DEACP and colistin reduced the CFU by 0.84 Log_{10} fold.

Figure 3.19 – Results of mouse keratitis model. CFUs obtained from excised mouse corneas upon treatment with 4 – 400 µg/mL of colistin (a). Overview of CFU results (b), and the corresponding results of three independent replicates (c – e) upon treatment with saline, 1 µg/mL imipenem (IMIP), 200 µM DEACP and/ or 40 µg/mL colistin. Each data point represents CFU from one cornea, suspended in 1 mL of saline, and error bars represent the standard error of the mean.

Variation in the results were observed within each independent experiment (Figure 3.19c – e). Within each independent experiment, groups treated with

imipenem, DEACP and colistin showed lower CFU than groups treated with imipenem alone ($\log_{10}$ change of independent experiments -0.47 ± 0.31), imipenem and DEACP ($\log_{10}$ change -0.19 ± 0.04) or imipenem and colistin ($\log_{10}$ change -0.16 ± 0.02).

3.4 Discussion

In this study, the kinetics and factors affecting NO release from cephalosporin-linked NO donor prodrugs were evaluated in both cell free systems and using *P. aeruginosa* biofilms. Subsequently, the efficiency of the lead compound, DEACP, in dispersing biofilms was assessed in *in vivo* mouse infection models. *In vitro*, NO release from such compounds was found to be influenced by several factors, including the concentration of imipenem used and its duration of treatment. Subsequently, dispersal was affected by the concentration of the prodrug used. Furthermore, induction of β -lactamase was affected by the growth conditions of *P. aeruginosa*. While the combination of imipenem and DEACP treatment could induce dispersal *in vitro*, its effects on the clearance of *P. aeruginosa* biofilms in mouse infection models were unclear, likely due to the above-mentioned variables and mechanisms of *P. aeruginosa* against repeated NO treatment.

3.4.1 NO release and activity of cephalosporin-linked NO donor pro-drugs are a function of the half-life of its diazeniumdiolate component.

During the activation of cephalosporin-linked NO donor prodrugs, NO is released from the NONOate following cleavage of the β -lactam ring and release of the NONOate component from the hybrid compound (Barraud *et al.* 2012, Yepuri *et al.* 2013). Consistent with the mechanism of activation, the kinetics of NO release from these compounds were reflective of the half-lives of individual NONOates, with UW-2 showing the fastest rate of NO release (Figure 3.4). As with previous studies, the compounds were able to disperse established *P. aeruginosa* biofilms with similar trends, in that higher concentrations of NO donors used give rise to

higher extent of dispersal. Nevertheless, differences were observed between compound behaviours between published and current studies.

In the previous study, UW-4 – 6 were determined to be less stable as they generate 2 – 3 fold higher levels of NO upon addition to the buffer (Yepuri *et al.* 2013). However, in this study, UW-0, 1 and UW 4 – 6 displayed similar NO release profiles. The compounds had low rates of decomposition in the absence of added β -lactamase and reached a stable level of $\sim 1800 \mu\text{M}$ NO upon activation (Figure 3.4). This is likely due to batch to batch variation, which may occur when experimental designs do not follow true exact replicates in terms of time, pressure, temperature, etc (Mockus *et al.* 2015). Batch to batch variation may also be due to difference in storage conditions, as cephalosporins, which constitute part of, and account for the stability of the hybrid, are known to be affected by moisture and it is generally advised to be stored protected from moisture and light. (Chalmers and Dent 2006, Loftsson 2014, Mimura *et al.* 2002))

Under similar conditions, $100 \mu\text{M}$ of each compound was previously shown to disperse $> 70\%$ of the biofilms, while in the current study, $<50\%$ of the biofilms were dispersed using the same concentrations of the compound. This variation is expected and may be due to differences in growth conditions, as dispersal by NO had been shown to occur over a range of concentrations (Appendix A).

Lastly, UW3 showed the highest activity for dispersal in previous studies but displayed a much lower rate of NO release and overall detection of NO in the current study. Activity of UW-3 was also much lower than that of DEACP, which was unexpected given that DEA NONOate (of UW-0) theoretically releases 1.5 moles of NO per mole of the compound while Pen-pip NONOate (of UW-3) could release up to 2 moles of NO per mole of the diazeniumdiolate compound (Ramamurthi and Lewis 1997, Saavedra *et al.* 1999). This may in part be due to batch to batch variations in synthesis and storage as described above, as low dispersal by UW3 was in line with its low rate of NO release as seen in the NO

release kinetics. Further, lower yields had been reported during the synthesis of UW3 as compared to DEACP (39% vs 75% yield) (Yepuri *et al.* 2013), which may have contributed to the reduction in activity.

3.4.2 NO release and dispersal by DEACP requires β -lactamase induction by imipenem, which is affected by different factors

Prodrug activation occurred in the presence of β -lactamases, such as penicillinase (Figure 3.4, 3.7). This requirement for β -lactamase activity had been demonstrated, where the addition of commercial β -lactamase gave rise to bursts of NO release when measured amperometrically (Yepuri *et al.* 2013). Besides imipenem, NAG and NAM were evaluated for their ability to induce β -lactamase. Both compounds did not have any effects on β -lactamase production. This is likely because β -lactamase induction via the AmpG-AmpR-AmpC pathway in *P. aeruginosa* is specific for GlcNAc-anhydro-MurNAc-peptides and 1,6-anhydroMurNAc-oligopeptides, and is not promoted by partial components like NAG and NAM (Zeng and Lin 2013).

Imipenem induced β -lactamase expression when exposed to 0.5 – 2 $\mu\text{g/mL}$ of the antibiotic, with higher levels of β -lactamase activity at or above the MIC (1 $\mu\text{g/mL}$). However, higher β -lactamase activity did not correlate with increased dispersal (Figure 3.8). This is likely due to the bactericidal effects of imipenem, as dispersal by NO is an active process involving metabolic changes in live cells (Huynh *et al.* 2012). Cell death due to increasing concentrations of imipenem, could therefore reduce the extent of dispersal observed as dead cells are unable to upregulate processes such as the secretion of matrix-degrading enzymes and remained trapped within the biofilm matrix, which can be stained by crystal violet (Nijland *et al.* 2010, Rybtke *et al.* 2015). Further, treatment with imipenem has also been shown to repress flagellum-encoding genes, which are typically upregulated in dispersing cells, with repression more significant in young biofilms (Bagge *et al.* 2004). It should, however, be noted that while flagellum-

upregulation has been associated with dispersal events, the extent of its contribution is unclear, as non-motile bacteria are also able to undergo active dispersal (Kaplan *et al.* 2003, Yarwood *et al.* 2004).

In addition to the type of inducer and concentration of imipenem used, β -lactamase production is also influenced by the duration of treatment with imipenem, due to requirements for its induction and production. Together, the results indicated that β -lactamase activity was essential for dispersal with DEACP treatment. However, dispersal may be influenced by other factors independent of β -lactamase activity, such as bactericidal effects of the imipenem. While these factors are important for the study of the NO donor pro-drugs in a controlled setting, *P. aeruginosa* from CF patients commonly display high levels of β -lactamase, and de-repressed mutants with higher basal β -lactamase are amongst the most frequent isolated strains for this phenotype (Campbell *et al.* 1997, Ciofu 2003, Llanes *et al.* 2013). In addition, *P. aeruginosa* biofilms isolated from other sites, including blood, urine, and wounds, also display high resistance to β -lactam drugs due to varying resistance mechanisms (Gales *et al.* 2001, Suk *et al.* 2007). Further, mature *P. aeruginosa* biofilms exposed to β -lactam drugs are also known to maintain high levels of β -lactamase production, with a thousand-fold higher MIC than younger biofilms (Bowler *et al.* 2012). As such, it stands to reason that there would be effective NO release from the prodrugs even in the absence of prior imipenem treatment, if administered in a clinical context.

3.4.3 Growth conditions affect dispersal *in vitro*

Experimental procedures and conditions differ between the *in vivo* models, namely the mouse intraperitoneal foreign body infection and keratitis model, and between the *in vivo* and *in vitro* plate models used in this study (Table 3.3).

In the mouse intraperitoneal foreign body infection model, placement of a biofilm-coated silicone implant in the peritoneal cavity may lead to intra-abdominal infections and sepsis that contribute to morbidity and mortality (Lopez *et al.* 2011). Due to reasons as such, a less virulent *P. aeruginosa* MPAO1 strain was evaluated for use in these models (Klockgether *et al.* 2010). MPAO1 has been reported to be genetically different from PAO1-UW and was also more resistant to imipenem (Klockgether *et al.* 2010). Despite these differences, both *P. aeruginosa* MPAO1 and PAO1 strains dispersed similarly when treated with imipenem and DEACP, indicating that the MPAO1 strain was suitable for use in the evaluation of dispersal.

Following previously established protocols, implant biofilms were initially formed in PBS and evaluated *in vitro* for use in the mouse intraperitoneal foreign body infection model (Christensen *et al.* 2007, Christensen *et al.* 2013). This is unlike in *in vitro* models where biofilms were formed in M9GC medium. However, *in vitro*, a lack of dispersal response to both S150 and DEACP with imipenem was observed for biofilms on implants developed in PBS (Figure 3.14). It was hypothesized that this was due to the cells in PBS being in a starved state, which is correlated with reduced ATP levels, lower metabolic rate and reduced protein synthesis (Cozzone 1981, Givskov *et al.* 1994, Groat *et al.* 1986, Huynh *et al.* 2012, Murray *et al.* 2003, Reeve *et al.* 1984). Contrary to expectations, relative ATP levels of cells grown or resuspended in M9GC or PBS were similar, with PBS resuspended cells displaying a slightly higher ATP level. Yet, in support of the hypothesis and consistent with reduced protein synthesis during starvation, no induction of β -lactamase activity by imipenem was observed for cells resuspended in PBS. Therefore, it is likely that the ATP levels were not reflective of the metabolic state of the cell, as actively growing cells were shown to display lower ATP levels due to consumption of ATP during protein synthesis (Schneider *et al.* 2002, Schneider and Gourse 2004).

Based on the expectation that dispersal would be limited for biofilms developed in PBS, the mouse intraperitoneal foreign body infection was first carried out

with biofilms grown in M9GC medium. High mortality was observed, even with the use of a lower virulence *P. aeruginosa* MPAO1 strain. As DEACP was not cytotoxic at five times the concentration used for the implant infection (Figure 3.12), mortality was likely to be due to a higher bacterial load. Studies using similar models showed that biofilm clearance may be affected by bacterial load (Christensen *et al.* 2012). When a flat square silicon was implanted, the PAO1 bacterial load was low ($\sim 10^6$) and BALB/c mice were able to clear away the infections (Christensen *et al.* 2007). However, the use of tubular silicone implants, as in this study, gave rise to higher bacterial load, leading to abscess formation and significantly higher bacteria counts (Christensen *et al.* 2012). Later experiments carried out with biofilms developed in PBS did not result in mortality, despite the implants having similar initial CFU as those grown in M9GC medium before implantation (data not shown). As such, in addition to the higher bacteria load, differences in virulence depending on culture medium composition and conditions may explain higher mortality in earlier experiments (Filiatrault *et al.* 2006, Ketola *et al.* 2016, Shepard and Gilmore 2002).

For intraperitoneal foreign body infection model carried out with biofilms developed in PBS, samples treated with either S150 or a combination of IMIP and DEACP displayed higher CFUs in both the spleen and implants than their respective controls after 24 h. Higher CFUs from the spleen samples of groups treated with NO donors or the prodrug was not unexpected, given that the spleen acts as a phagocytic filter for the removal of bacteria from the bloodstream, where dispersal was presumed to result in higher bacteria counts and increased bacteria load (Bohnsack and Brown 1986, Li *et al.* 2014). However, the results differ from previous studies in two ways. Firstly, massive dispersal of *P. aeruginosa* from the implant site was observed in previous study (Christensen *et al.* 2013), while an increase in implant CFU was recorded in the experiments reported here. Secondly, dispersal resulted in only a transient increase in bacteria accumulation in the spleen of mice. Bacteria counts within the spleen declined after 5 h to reach levels comparable to that of undispersed samples, and no discomfort was

observed in the mice by the latest time point of 15 h (Christensen *et al.* 2013). One explanation may be the method used for inducing dispersal. In the previous study, dispersal was induced directly through induction of a plasmid encoded PDE, leading to constitutive decreases in cdGMP levels. However, in the current study, dispersal was induced using NO. While NO is known to exert its effects on dispersal through regulation of cdGMP and PDE activity, specific pathway that is altered by perception of NO is not well understood (Barraud *et al.* 2009a). Thus, it is not clear if PDE induced dispersal alters the expression of the same effectors as NO induced dispersal. For example, dispersal due to external stimuli is also thought to proceed through transient changes involving initial increases in cdGMP levels (Basu Roy and Sauer 2014, Nair *et al.* 2016). Furthermore, the reactivity of NO suggests that dispersal effects exerted by NO may be short-lived. It is hence possible that, unlike in the previous study, the implant biofilm could have reformed following dispersal. Other biofilms had, too, been shown to recover rapidly, within 24 h after mechanical disruption (Mancl *et al.* 2013). In addition, despite the benefit that dispersal increases the susceptibility of biofilms to antibiotics, the process itself is associated with pathogenicity (Li *et al.* 2014), with dispersed cells being comparatively more virulent than their planktonic counterparts (Chua *et al.* 2014). Hence, there is a limited window in which antimicrobial treatment following dispersal would be effective in eliminating both dispersed and biofilm cells (Mancl *et al.* 2013). In contrast, in this study, antibiotics were not administered following dispersal due to limitations of the model. The increased bacteria load following dispersal might hence contribute to the increase in bacteria recovered from the implant.

3.4.4 Contribution of other factors to effectiveness of treatment *in vivo*

Because of the limitations and mortality of the mouse intraperitoneal foreign body infection model, the compounds were subsequently evaluated using the mouse keratitis model, where infection is localized to the cornea and can be easily

accessed and treated. In this model, the effective concentration of colistin was found to be higher than when evaluated *in vitro*. This is not uncommon, as other studies had indicated differences in efficacy of antibiotics *in vitro* and *in vivo* (van Ogtrop *et al.* 1990). Drug application to the cornea may also be affected by pharmacokinetic factors like drug penetration, or physiological factors such as blinking, tear turnover or drainage into the nasal cavity (Bourlais *et al.* 1998, Chaurasia *et al.* 2015, Zhang *et al.* 2004).

With the keratitis model, a combination therapy of imipenem, DEACP and colistin was generally more effective than treatment single compounds (imipenem) or two compounds (imipenem and colistin or DEACP). However, the extent of such reduction in CFU differed in each independent experiment, with no significant reductions between each treatment. The lack of effects may be due to host or pharmacokinetic factors, such as blinking, tearing and drug penetration, as previously mentioned. Changes in cdGMP levels due to NO had also been reported to influence immune response due to phagocyte recruitment to the site of infection and cytokine production (Yam *et al.* 2017). While the exact NO levels released from the added compounds at the site of infection is not known, one could expect that variation in NO release and host response may also account for the differences observed. Repeated dosing with NO, may also promote the expression of genes involved in resistance to NO treatments in remaining biofilms, such as *fhp* (Zhu *et al.* 2017), as *in vitro* experiments indicated that multiple dosing with NO reduces the extent of dispersal in the wildtype but not in the *fhp* transposon mutant (Figure 3.18).

3.5 Conclusions and future studies

The use of cephalosporin-linked NO donor pro-drugs enables specificity in targeting NO to bacterial biofilms. However, such specificity comes with a trade-off, as several factors involved in the multistep activation of the prodrug may affect the outcome of dispersal. *In vitro*, the pro-drugs could effectively disperse

P. aeruginosa biofilms with a one-time treatment and had been shown to promote eradication of both planktonic and biofilm cells. However, results *in vivo* did not corroborate with the *in vitro* results, likely due to differences in experimental conditions and influences of host factors. This is further complicated by responses of *P. aeruginosa*, which may also develop strategies against dispersal from further NO treatment. While there is potential in the therapeutic application of such compounds, future challenges lie in optimization of each step in the activation of the prodrug, or changes in drug design, to improve the effectiveness of the compound. The latter of which is currently being worked on and will be elaborated in the section below.

At present, cephalosporin-NO donor compounds were designed for targeted NO release in mind. As such, currently available compounds display low bacterial toxicity, i.e. the cephalosporin component does not have any bactericidal effects (Table 3.5). In addition, the compounds require prior induction of β -lactamase for activity. In the context of clinical biofilms, which generally produces elevated levels of β -lactamase, such means of drug activation is unlikely to pose a problem. However, for evaluation of drug activity *in vivo* in a controlled setting, this additional step complicates the process of drug activation.

Several new cephalosporin-linked NO donor compounds have been designed and is currently being evaluated to replace DEACP as the lead compound (Appendix C). Unlike compounds of the previous group, these new compounds had been designed for specificity of activation by PBP. The hypothesis that cephalosporin-linked NO donor compounds could be activated by PBPs is based off observations in previous studies, in which NO release from DEACP was detected amphiphotically when cell extracts from an *E. coli* strain not known to produce β -lactamase was added (Barraud *et al.* 2012). Specificity of the new compounds for activation by PBPs can be achieved through modification of side chains present on the β -lactamase moiety. In addition, the new compounds display varying degrees of bactericidal activity (Appendix C). This would enable dual activity of both dispersal of biofilm and killing of bacterial cells simultaneously

and is viable as NO release from PBP activated compounds are not dependent on β -lactamase, the induction of which is dependent on the presence of live bacteria. In theory, the use of such new compound would increase synergistic effects and further promote biofilm eradication when used in the presence of another antibiotic following biofilm dispersal. Characterization and evaluation of the activity from these new compounds is ongoing can be found in the appendix.

4. Mechanisms of NO induced dispersal

4.1 Introduction

Nitric oxide (NO) is a ubiquitous signalling molecule that acts as a signal for biofilm dispersal in many bacteria species, including *Pseudomonas aeruginosa* (Barraud *et al.* 2006). In several bacteria, such as *Legionella pneumophila*, *Shewanella woodyi* and *Shewanella oneidensis*, H-NOX (Heme-Nitric Oxide/Oxygen binding domain) based sensor proteins are commonly involved in the sensing of NO (Carlson *et al.* 2010, Liu *et al.* 2012, Plate and Marletta 2012a). These proteins are homologous to the known mammalian NO sensor, soluble guanylate cyclase, and may regulate downstream activities via fused modular methyl-accepting chemotaxis domains, modulation of cyclic-di-GMP (cdGMP) levels or interactions with histidine kinases (Boon *et al.* 2006, Cutruzzola and Frankenberg-Dinkel 2015). However, *P. aeruginosa* is not known to encode any H-NOX proteins, and the pathway through which *P. aeruginosa* senses and responds to NO for dispersal is not well understood.

In general, the biofilm dispersal event is correlated with a decrease in intracellular cdGMP levels. The cdGMP levels are modulated by the activity of two enzymes, namely, diguanylate cyclase (DGC) containing a GGDEF domain that synthesizes cdGMP, and phosphodiesterase (PDE), which contains an active EAL or HD-GYP domain and hydrolyses cdGMP to pGpG (Jenal and Malone 2006). In *P. aeruginosa*, the exogenous addition of NO has been found to induce PDE activity in cell-free extracts, suggesting that post-translational activation of PDE activity could contribute to reduced cdGMP levels, leading to NO induced dispersal (Barraud *et al.* 2009a).

The *P. aeruginosa* genome encodes multiple proteins with one or both GGDEF or EAL domains (Kulesekara *et al.* 2006). Several di-domain proteins consisting of active or inactive GGDEF and EAL domains, including RbdA, DipA, NbdA and MucR, had been implicated in NO induced dispersal, as mutation of these

genes were found to abolish the NO-induce dispersal phenotype (An *et al.* 2010, Li *et al.* 2013a, Roy *et al.* 2012). Furthermore, the exogenous addition of NO was also found to increase transcription of *nbdA*, coding for an active PDE, which might contribute to NO induced dispersal in addition to the previous hypothesis that NO induced dispersal may be post-translationally regulated (Li *et al.* 2013a).

RbdA, DipA, NbdA and MucR also contain known sensor domains in addition to their GGDEF and EAL di-domains. RbdA and DipA contain a PAS sensor domain, which is known to be able to bind diatomic gases, e.g. oxygen, carbon monoxide and NO, depending on the prosthetic group associated with the PAS domain (Gilles-Gonzalez and Gonzalez 2004). Further, RbdA was previously shown to play a role in oxygen sensing (An *et al.* 2010). Sequence alignment of RbdA to its homolog, FixL, also indicated that RbdA may bind a heme prosthetic group, suggesting that RbdA may have the potential to function as a sensor of NO (An *et al.* 2010). NbdA and MucR, on the other hand, contain a MHYT domain, which is a seven-transmembrane copper binding domain that was proposed to similarly sense oxygen, carbon monoxide and NO (Galperin *et al.* 2001, Li *et al.* 2013a). Taken together with idea that post-translation modification may contribute to NO induced dispersal, it was hypothesized that these proteins may act as sensors of NO and modulate their PDE activity to influence intracellular cdGMP levels and facilitate the dispersal process.

Despite the hypotheses that these proteins may sense NO, no direct evidence of such activity has been demonstrated and the mechanisms of NO induced dispersal is still not well understood for *P. aeruginosa*. In this study, two of proteins, RbdA and DipA were expressed and purified to assess if they can bind NO directly and the effect of NO addition on protein activity. The two proteins were not found to be involved in direct NO sensing, suggesting that NO-induced dispersal may involve other mechanisms. Based on available literature, several other genes or targets hypothesised to be involved in NO sensing or NO dispersal were identified for screening to shed light on their roles in NO-induced dispersal. The NO dispersal response of transposon mutants of the relevant targets was

determined to evaluate their role in NO induced dispersal. Mutants of genes previously implicated in the process, including *mucR*, *dipA* and *nbdA*, were however, not found to be defective in NO induced dispersal in our model, likely due to redundancies in DGCs and PDEs encoded in *P. aeruginosa*.

Figure 4.1 – Summary of potential mechanisms of biofilm dispersal specifically driven by NO. Processes in green boxes are specific for *P. aeruginosa* while processes in the grey box represents the mechanism of NO-induced dispersal in other bacteria *spp*. Besides dispersal, NO plays a role in denitrification processes (a). Inhibition of respiration is also known to result in biofilm dispersal (b). In general, a reduced cdGMP level via transcriptional and/or post-translation regulation of PDE activity is associated with dispersal, although studies indicate that the process may also involve a transient increase in cdGMP levels (c). Deletion of genes involved in cdGMP regulation, specifically *nbdA*, *mucR*, *rbdA* and *dipA*, abolishes the NO dispersal phenotype (d). For *nbdA*, the process involves upregulation of gene transcription (e). A potential mechanism for DipA and RbdA had been proposed based on studies on glutamate induced dispersal, although it is unclear if the same process is involved in NO-induced dispersal (c). In addition, NO-induced dispersal in *P. aeruginosa* can proceed through *nosP* and NahK through unknown mechanisms (f). In this study, based on the hypothesis that PDE activity is regulated post-translationally, we purified RbdA and DipA to determine if these proteins may act as direct sensors of NO. As this does not appear to be the case, studies were carried out to screen genes involved in the processes presented in the figure to determine other mechanisms through NO may regulate the process.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

All bacterial stains (Table 4.1, Appendix B for list of transposon mutant strains) were maintained on LB10 agar supplemented with antibiotics as needed. *E. coli* BL21 (DE3) Rosetta T1^R strains were selected with 34 µg/mL chloramphenicol, while 30 µg/mL kanamycin was used for the maintenance of plasmids. *P. aeruginosa* transposon mutants were selected using 60 µg/ml of tetracycline. Unless otherwise stated, overnight cultures were obtained by inoculation in 10 mL LB10 medium in 50 mL falcon tube at 37°C with 200 rpm shaking.

4.2.3 Biofilm microplate assay

The biofilm microplate assay was carried out as previously described in Chapters 2 and 3. Briefly, overnight *P. aeruginosa* strains were diluted 200 times in M9GC medium (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.04% w/v glucose, 0.2% w/v casamino acid, pH 7.0). One mL of the diluted culture was added into a well of a 24 well plate, which was further incubated at 37°C with 200 rpm shaking for 6 h. At t = 6 h, the NO donor compound, spermine NONOate (S150) (136587-13-8, Cayman Chemicals), was added into NO-treated groups to a final concentration of 200 µM for 30 min to induce dispersal. Biofilm biomass was quantified by crystal violet (CV) staining and the extent of dispersal was evaluated as previously described. For transposon mutant and deletion mutant screening, the extent of dispersal of the mutant was subsequently compared to that of the MPAO1 WT or other relevant strain, evaluated under the same conditions.

$$\text{Relative extent of dispersal} = \frac{\% \text{ dispersal by mutant}}{\% \text{ dispersal by WT}}$$

A value < 1 indicates that the mutant dispersed less than the WT while a value > 1 indicates that mutant dispersed more than the WT. Data represent the average OD₅₅₀ and extent of dispersal, calculated within each independent study, and comprised of three biological replicates. Differences in the extent of dispersal between the mutants and WT were compared using ANOVA with multiple comparisons to the control group (MPAO1 WT) using Dunnett's test ($\alpha = 0.05$) in Graphpad prism V7.02.

4.2.3 Sample preparation for RNA isolation

Sample preparation, RNA isolation and RNA-seq data analysis were carried out by Zhu XY. For RNA isolation and sequencing, planktonic and biofilm cells were grown using the same method as described in the biofilm plate assay, in M9G medium (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4% w/v glucose, pH 7.0). Untreated planktonic and biofilm cells were collected at $t = 6$ h. For NO-treated samples, the plates were incubated for 5 h 45 min before the spent medium was removed. The cells were then washed with PBS before treatment with fresh M9 medium with 100 μ M S150 for 15 min. Biofilms and dispersed cells were collected at $t = 6$ h following treatment with the NO donor. The experiment was performed in triplicates.

4.2.4 RNA extraction, sequencing and data analysis

Total RNA was extracted from samples using a Direct-zolTM RNA MiniPrep kit (Zymo Research). A Turbo DNA-free rigorous protocol (Ambion) was used for DNase treatment. RNAs were concentrated and purified using the RNA Clean & ConcentratorTM-5 kit (Zymo Research) and the integrity of the total RNA and amount of DNA contamination were assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit 2.0 Fluorometer (Invitrogen). mRNA data was analyzed using TopHat and Cufflinks softwares (Trapnell *et al.* 2012), with

sequence reads mapped onto the *P. aeruginosa* PAO1 reference genome (NCBI NC_002516.2). Reads with significant fold-change with respect to the relevant control groups ($\alpha < 0.05$), were presented.

4.2.5 PDE assay

PDE activity was determined using the PDE specific chromogenic substrate bis(p-nitrophenyl) (bis pNpp) as previously described (Barraud *et al.*, 2009). In the presence of PDE, bis pNpp is hydrolyzed to para-nitrophenol, which has a characteristic yellow color and absorbs strongly at 405 nm. PDE buffer (50 mM NaCl, 50 mM Tris-Base pH 8.0, 1 mM MnCl₂, 5 mM bis pNpp) was freshly prepared before each experiment. To assess the effect of NO on PDE activity, the NO donor, S150 was also added to PDE buffer to a final concentration of 200 μ M.

For experiments involving whole cell extracts, 30 mL of overnight PAO1 cultures was first centrifuged at $12\,000 \times g$. The cells were resuspended and lysed with 500 μ L B-PER reagent (90084, Thermo scientific). Following lysis, cells were centrifuged at $12\,000 \times g$ for 10 min. The supernatant was collected and filtered through a 0.22 μ m filter to remove unlysed cells. Filtered cell extracts were then mixed with PDE buffer. One hundred microliters of the mixture were then aliquoted into a 96 well plate. For experiments involving purified proteins, the purified protein constructs were added to 100 μ L of PDE buffer on a 96 well plate to a final concentration of 0 - 128 μ g/mL of proteins. At least duplicates of each experimental group were prepared.

The plates were then incubated at 37°C with 100 rpm shaking in the dark. PDE activity was determined spectrophotometrically using a microtiter plate reader and measuring absorbance at 405 nm every 30 min. Analysis of PDE activity based on the rate of change in absorbance over time was determined via linear regression, while correlation between changes in absorbance and time or

concentration of protein used was determined via F-tests in Graphpad prism V7.02.

4.2.6 Small scale protein expression

Protein expression and purification were carried out at the Protein Production Platform (PPP) at NTU. All plasmids and primers used for ligation-independent cloning are listed in Tables 4.1 and 4.3, respectively. Transformed clones of *E. coli* BL21-DE3 Rosetta strains were grown in 1 mL of terrific broth (TB) (1.2% w/v trypton, 2.4% w/v yeast extract, 0.4% v/v glycerol, 0.17 M KH₂PO₄, 0.72 M K₂HPO₄) in 96 deep well plates at 37°C to an OD₆₀₀ of 2 – 3, at which time, the temperature was lowered to 18°C and protein production was induced with the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (I6758, Sigma Aldrich) overnight. The presence of the target protein was subsequently verified using SDS-PAGE. Protein expression was then scored to reflect the levels of soluble and purifiable proteins from the clone, with a score of 1 indicating that target protein was present but significantly weaker than the background while a maximum score of 4 indicating that the target protein was present at levels significantly higher than the background. A score of 0 indicates that no target protein was present. Scores of 0 – 2 were considered low expression scores while scores of 3 and above were high expression scores.

4.2.7 Large scale protein expression

Transformed clones were inoculated overnight at 37°C with 200 rpm shaking in TB supplemented with 8 g/L of glycerol (TBG) and appropriate antibiotics. To scale up protein production of target proteins with high expression scores (based on small scale expression), 20 mL of the overnight culture was used for further inoculation of 750 mL of TBG supplemented with the appropriate antibiotics and 100 μL of antifoam 204 (A6426, Sigma Aldrich). Two times the volume (40 mL)

of starter culture and medium volume (1.5 L) were used for cultivation of *E. coli* expressing proteins that have low expression scores, with proportional increases in antibiotics and antifoam 204 supplied. The inoculated cultures were incubated at 37°C in the LEX system (Harbinger Biotech), with aeration and agitation via bubbling of filtered air through the cultures. The incubation temperature was reduced to 18°C when the OD₆₀₀ of culture was ~2. Following which, protein expression in the cultures were induced with 0.5 mM of IPTG for 60 min. Protein expression was allowed to continue overnight under the same conditions. Cells were harvested the following day by centrifugation at 6000 × g, 4 °C for 10 min. The supernatant was removed and the cell pellet was stored at – 80°C until use.

4.2.8 Protein purification

Fresh or frozen cell pellets from large scale protein expression were resuspended in ~ 3 × pellet volume of buffer A (20 mM HEPES, pH 7.5, 300 mM NaCl, 5% v/v glycerol, 0.5 mM TCEP), with 25 µL of protease inhibitor cocktail (P8849, Sigma Aldrich) added. The suspended cells were vortexed until a homogenous sample was obtained, and was lysed via sonication at 35% amplitude with 3/6 s on/off cycle for 30 min. The lysed sample was then centrifuged at maximum speed for 45 min at 4°C for separation of the cell debris from the protein fraction. The supernatant was subsequently collected and filtered through a 0.2 µm filter before incubation and binding to Ni-NTA beads (25214, Thermo Scientific), which had been previously equilibrated with 2 column volumes of buffer A, for 1 h at 4°C. Proteins were eluted using a step-gradient method with buffer A containing increasing concentration of imidazole (10 mM – 750 mM). The eluted samples were collected and used for SDS-PAGE followed by Coomassie blue staining to identify fractions containing the protein of interest.

Fractions containing the protein of interest was then pooled and concentrated at 4°C using Vivaspin® 6 concentrators (19-49 kDa – 10K MWCO, >50 kDa – 30K

MWCO) to a volume of 1 – 5 mL. Concentrated samples were used for size-exclusion chromatography (SEC) with the AKTA system using a HiLoad 16/60 Superdex 200 prep grade column. SEC was carried out at 4°C using buffer A. Fractions from SEC, containing the protein of interest, were again pooled to obtain the highest possible protein purity and concentrated at 4°C using the same Vivaspin® 6 concentrator. Protein concentrations were determined by measuring absorbance at 280 nm on Nanodrop-2000 (Nano-drop technologies). The final protein purity was assessed by SDS-PAGE. The final protein batch was then aliquot into smaller fractions, flash frozen in liquid nitrogen and stored at – 80°C until used.

4.2.9 UV-Vis spectroscopy for detection of heme binding

Absorption spectra were measured using 2.5 – 10 µM of protein in buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 5 % Glycerol and 0.5 mM TCEP at 23°C in a capped quartz cuvette. The spectrum was recorded with a UV-Vis spectrophotometer (UV1800, Shimadzu) via the UV Probe software using a medium scan speed and interval of 0.1 nm.

Table 4.1 – Bacterial strains and plasmids used for protein expression and purification.

Strain or plasmid	Relevant genotype/ description	Source
<i>Escherichia coli</i>		
BL21(DE3) Rosetta T1R	F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B - m _B -) <i>gal dcm</i> (DE3) pRARE (Cam ^R) T1 ^R	PPP platform, NTU SBS
Plasmids		
pNIC28-Bsa4	Kan ^R , vector for cloning and cleavable N-terminal 6 × His fusion protein expression	(Savitsky <i>et al.</i> 2010); Accession no. EF198106
pNIC28-CH2	Kan ^R , vector for cloning and C-terminal 6 × His fusion protein expression	(Savitsky <i>et al.</i> 2010); Accession no. EF199843
pNIC28-GST	Kan ^R , vector for cloning and N-terminal GST/6 × His fusion protein expression. GST/6 × His tag separately cleavable	(Savitsky <i>et al.</i> 2010);
pNIC28-Bsa4- <i>bifA</i>	Plasmid for overexpression of full length BifA	This study
pNIC28-Bsa4- <i>bifA</i> (184-686)	Plasmid for overexpression of BifA with truncated transmembrane region	This study
pNIC28-Bsa4- <i>rbdA</i> (1-818)	Plasmid for overexpression of full length RbdA	This study
pNIC28-Bsa4- <i>rbdA</i> (233-818)	Plasmid for overexpression of RbdA with truncated transmembrane region	This study
pNIC28-Bsa4- <i>rbdA</i> (246-818)		This study
pNIC28-Bsa4- <i>rbdA</i> (233-800)		This study

Table 4.1 (Cont'd)

pNIC28-Bsa4- <i>rbdA</i> (246-800)		This study
pNIC-CH2- <i>rbdA</i> (233-818)		This study
pNIC-CH2- <i>rbdA</i> (233-800)		This study
pNIC-CH2- <i>rbdA</i> (246-818)		This study
pNIC-GST- <i>rbdA</i> (246-800)		This study
pNIC28-Bsa4- <i>rbdA</i> (367-800)	Plasmid for overexpression of RbdA GGDEF/EAL	This study
pNIC28-CH2- <i>rbdA</i> (367-800)		This study
pNIC28-Bsa4- <i>rbdA</i> (231-366)	Plasmid for overexpression of RbdA PAS	This study
pNIC28-Bsa4- <i>dipA</i> - (1-899)	Plasmid for overexpression of full length DipA	This study
pNIC28-Bsa4- <i>dipA</i> (1-462)	Plasmid for overexpression of DipA GAF/PAS	This study
pNIC28-Bsa4- <i>dipA</i> - (174-899)	Plasmid for overexpression of DipA GAF/PAS/GGDEF/EAL	This study
pNIC28-Bsa4- <i>dipA</i> - (174-885)		This study
pNIC28-CH2- <i>dipA</i> (174-899)		This study
pNIC28-Bsa4- <i>dipA</i> - (355-899)		This study
pNIC28-Bsa4- <i>dipA</i> - (355-885)		This study

Table 4.1 (Cont'd)

pNIC28-CH2- <i>dipA</i> (367-899)		This study
pNIC28-Bsa4- <i>dipA</i> - (462-899)	Plasmid for overexpression of DipA GGDEF/EAL	This study
pNIC28-CH2- <i>dipA</i> (462-899)		This study
pNIC-GST- <i>dipA</i> (462-899)		This study
<i>Pseudomonas aeruginosa</i>		
PAO1	WT	ATCC BAA-47
PAO1 UT	WT lab strain	(Arai <i>et al.</i> 2014)
QxAaS2	Suppressor mutant of mutant with deletion of <i>cyoABCDE</i> , <i>cioAB</i> , <i>ccoN1O1Q1P1</i> , and <i>ccoN2O2Q2P</i>	(Arai <i>et al.</i> 2014)
QxBo	Deletion of <i>cioAB</i> , <i>coxBA-coxC</i> , <i>ccoN1O1Q1P1</i> , and <i>ccoN2O2Q2P2</i>	(Arai <i>et al.</i> 2014)
QxCb1	Deletion of <i>cyoABCDE</i> , <i>cioAB</i> , <i>coxBA-coxC</i> , and <i>ccoN2O2Q2P</i>	(Arai <i>et al.</i> 2014)

Table 4.2 – Primer pairs and sequences used in this study.

Constructs	Primers	Primer sequence (5' → 3')
BifA (1 – 686)	F5531	TACTTCCAATCCATGAAACTGGACTCCCGA CACAG
	R5519*	TATCCACCTTTACTGTCAGGGCCGTTTCGCT GCTGG
BifA (184 – 686)	F5530	TACTTCCAATCCATGAAGCCGCTGTCGAAG ATCAT
	R5519*	
RbdA (1 – 818)	F5534	TACTTCCAATCCATGATGAGGCAGAACCG GACTC
	R5520	TATCCACCTTTACTGTCACCGGAGGTTCTG TCCCAG
RbdA (233 – 800)	F6150	AGAGGAGATAATTAATGCGCCAGGAAGTG GAGAACG
	R6189*	AATGGTGGTGATGATGGTGCGCCGGAGGC TGGCGACTGAA
RbdA (367 – 800)	F6198*	AGAGGAGATAATTAATGATGGCGCGCCTG TCCTGG
	R6189*	
RbdA (367 – 818)	F6198*	
	R6190	AATGGTGGTGATGATGGTGCGCCCGGAGG TTCTGTCCCAG
RbdA (231 – 366)	F6238	TACTTCCAATCCATGATGCAGCGCCAGGA AGTGG
	R5600	TATCCACCTTTACTGTCAGTGCAGGGTGCG GCAGG
DipA (1 – 899)	F5620*	TACTTCCAATCCATGATGAAAAGTCATCCC GATGCC
	R5601	TATCCACCTTTACTGTCACAGCTTGTTCTG CGTGATGT
DipA (1 – 462)	F5620*	
	R6267	TATCCACCTTTACTGTCAGTACTGGCGCTC GCGGG

Table 4.2 (Cont'd)

DipA (174 – 899)	F6142	AGAGGAGATAATTAATGACCCAGCAGAAC CTGATCG
	R6128*	AATGGTGGTGATGATGGTGCGCGTGCAGG GTGCGGCAGG
DipA (355 – 899)	F6143	AGAGGAGATAATTAATGCTGGCCCAGCAG CACATC
	R6128*	
DipA (462 – 899)	F6145	AGAGGAGATAATTAATGAGCGCCAGCGCC TTCCTG
	R6128*	

* Primer ID of sequences used in multiple constructs. Sequences are only entered for the first ID listed.

4.3 Results

4.3.1 Protein expression and purification

Three proteins, BifA, DipA and RbdA, were selected for protein expression. These proteins were to be purified and evaluated for any direct NO interactions that may suggest their role as NO sensors. DipA and RbdA were selected due to their reported involvement in NO induced dispersal (Roy *et al.*, 2012), and the presence of a PAS domain, which had been reported to be able to bind NO. While both NbdA and MucR were also hypothesized to be involved in NO-induced dispersal, their expression was difficult considering the transmembrane nature of predicted NO binding MHYT domain. Further, NO is membrane-permeable and most known NO sensors are soluble (Plate *et al.*, 2012).

BifA was selected as a positive control, as it contains the same domains of GGDEF and EAL as DipA and RbdA. Like DipA and RbdA, BifA is a functional PDE but not DGC. BifA does not contain a PAS domain, which is hypothesized to play a role in NO sensing and BifA had not been previously implicated in NO induced dispersal in *P. aeruginosa*.

Constructs that were successfully cloned into *E. coli* BL21(DE3) were screened for soluble protein expression using SDS-PAGE. Designed protein constructs and their expression levels are reflected in Figure 4.2, with protein expression of soluble constructs on SDS-PAGE gels shown in Figure 4.3.

Full length BifA and RbdA (B1 and R1) were well expressed (Figure 4.3). However, they were not used for downstream purification due to difficulties in working with transmembrane proteins. Instead, truncated constructs containing all the relevant domains were cloned and expressed. B2, a truncated N-terminal His-tagged BifA, comprised of amino acids from positions 184 – 666, containing both GGDEF and EAL domains, but not the transmembrane domain, was expressed strongly (score = 3) and selected for large-scale protein expression and purification.

RbdA constructs (R2–R9) containing GGDEF and EAL domains, but lacking the transmembrane region were not well expressed regardless of construct length or affinity tags used. The proteins were either insoluble (R2–R6, R8–R9) or not expressed well against background protein expression (R7) (Figures 4.2 and 4.3). R7, a C-terminal His-tagged RbdA construct containing amino acids from position 233 – 800, displayed a faint band that was weaker than the background protein expression levels (score = 1) (Figure 4.3). Similarly, RbdA constructs without the PAS and transmembrane domains (R10 and R11) were weakly expressed (score = 2) in the soluble fraction. R12, consisting of only the PAS domain had better expression (score = 4) compared to other truncated constructs of RbdA. However, without the enzymatic GGDEF and EAL domains, the construct was not applicable for evaluation of PDE activity with NO.

Unlike BifA and RbdA, full length DipA (D1) was not expressed in the soluble fraction. Soluble expression of truncated proteins of DipA containing all domains (D5) or lacking only the GAF domain (D8) was improved using a C-terminal His-tag (Figure 4.1). Aside from D2, which contains only the DipA GAF and PAS domain, no other constructs of DipA were suitably expressed in the soluble fraction with an N-terminal His-tag. Solubility and expression of DipA proteins without both GAF and PAS domains were also improved in the presence of a large soluble protein such as glutathione-S-transferase (D11) (Figure 4.2). However, since a large GST protein may interfere with downstream experiments, only soluble constructs with a C-terminal His-tag were selected for large scale protein expression and purification.

Based on the small-scale protein expression data, B2, R7, R12, D5 and D8 were selected for large scale protein purification. A small volume of the purified protein batch was used for SDS-PAGE to assess the purity of the purified proteins (Figure 4.3). B2 and R12 were relatively pure, containing higher amounts of the protein construct of interest. However, samples of R7, D5 and D8 contained contaminating proteins in similar amounts as the protein construct of interest. Hence, the final protein samples for R7, D5 and D8 consist of a mixture of

proteins, for which the final protein concentration may not be reflective of actual concentrations of R7, D5 or D8 present in the sample.

	Construct ID	Construct	AA	Domains															
BifA	BifA																		
	B1	Full length	1-686																
	B2	ΔTM	184 – 686																
RbdA	RbdA																		
	R1	Full length	1 – 818																
	R2	ΔTM	233 – 818																
	R3	ΔTM	246 – 818																
	R4	ΔTM	233 – 800																
	R5	ΔTM	246 – 800																
	R6	ΔTM	233 – 818																
	R7	ΔTM	233 – 800																
	R8	ΔTM	246 – 800																
	R9	ΔTM	246 – 800																
	R10	ΔPAS	367 – 800																
	R11	ΔPAS	367 – 800																
	R12	PAS	231 – 366																
DipA	DipA																		
	D1	Full length	1 – 899																
	D2	GAF/PAS	1 – 462																
	D3	Δ ₁ – 173	174 – 899																
	D4	Δ ₁ – 173	174 – 885																
	D5	Δ ₁ – 173	174 – 899																
	D6	ΔGAF	355 – 899																
	D7	ΔGAF	355 – 899																
	D8	ΔGAF	355 – 899																
	D9	ΔGAF/PAS	462 – 899																
	D10	ΔGAF/PAS	462 – 899																
	D11	ΔGAF/PAS	462 – 899																
Legend	Protein domains and tags																		
Expression levels																			

Figure 4.2 – List of constructs and construct design, including cloned regions and affinity tags used, of BifA, DipA and RbdA. Domains present in each protein are displayed as coloured blocks on the first row of each section. Protein constructs are shown below the full-length proteins with the blocks corresponding to the domains included in the construct. Constructs, excluding the affinity tags used, are displayed as red or green to reflect a lack or presence of soluble protein expression respectively.

Figure 4.3 – Expression of soluble constructs of BifA, DipA and RbdA, and purified samples of B2, R7, R12, D5 and D8. Arrows indicate the band corresponding to the protein of interest against a background of soluble proteins from *E. coli*.

4.3.2 PDE activity of purified proteins

Figure 4.4 – PDE activity of 0 – 16 µg/mL proteins. Changes in absorbance at 405 nm over 3 h due to hydrolysis of bis-pNpp by PDE at 0 – 16 µg/mL of BifA B2 (a), RbdA R7 (b), DipA D5 (c) and DipA D8 (d). Data represent values from at least two replicates whereas error bars represent the standard deviation of the mean.

The PDE activity of the proteins were first determined to ensure that the purified proteins retain their activity, and to determine a suitable concentration for detection of any changes in protein activity following addition of NO. PDE activity could be observed as an increase in absorbance at 405 nm overtime. All constructs evaluated (B2, R7, D5 and D8) displayed PDE activity, indicating that they were functional. Within the detection limits of the assay, at concentrations of less than 6 µg/mL of BifA B2 and 16 µg/mL of RbdA R7, DipA D5 and D8 showed no detectable PDE activity (Figure 4.5). D5 displayed biphasic activity (Figure 4.5 and 4.7), with higher activity from 0 – 30 min ($0.277 \pm 0.0016 \text{ h}^{-1}$ at 128 µg/mL, fig 4.5), but reduced activity from 30 min onwards ($0.0799 \pm 0.0063 \text{ h}^{-1}$ at 128 µg/mL, fig 4.5). The rate of bis-pNpp hydrolysis was highly correlated

to the concentration of protein used for B2 from 1 – 12 $\mu\text{g/mL}$ ($R^2 = 0.982$, $p = 0.009$) and from 8 – 128 $\mu\text{g/mL}$ for R7, D5 and D8 ($R^2 = 0.991$ ($p = 0.0004$), 0.986 ($p = 0.0040$) and 0.956 ($p = 0.0010$) respectively) (Figure 4.6). For R7, D5 and D8, rate of bis-pNpp hydrolysis was not found to correlate to the concentration of protein used from 1 – 4 $\mu\text{g/mL}$ ($p \geq 0.05$) (Figure 4.6). D8 was found to have higher activity, with a rate of change of absorbance due to bis-pNpp hydrolysis at 0.200 h^{-1} , while D5 and R7 had a rate of 0.119 and 0.111 h^{-1} respectively.

Figure 4.5 – PDE activity of 0 – 128 $\mu\text{g/mL}$ proteins. Changes in absorbance at 405 nm over 3 h due to hydrolysis of bis-pNpp by PDE at 0 – 128 $\mu\text{g/mL}$ of RbdA R7 (a), DipA D5 (b) and DipA D8 (c). Data represent values from at least two replicates whereas error bars represent the standard deviation of the mean.

Figure 4.6 – PDE activity against protein concentration. Rate of change in absorbance at 405 nm at 2 – 12 μg/mL (a), 8 – 128 μg/mL (b, d, f) and 1 – 8 μg/mL (c, e, g) of B2 (a), R7 (b, c), D5 (d, e) and D8 (f, g). Changes in rate against concentration were linearly correlated from 2 – 12 μg/mL for B2 and 8 – 128 μg/mL for R7, D5 and D8, but not at lower concentrations of 1 – 8 μg/mL of R7, D5 and D8, based on analysis with F-tests.

4.3.3 PDE activity in the presence of NO

NO induced dispersal is associated with a reduction in intracellular cdGMP concentrations. Therefore, it is possible that NO mediates this effect by binding to and modifying the cdGMP degrading activity of PDEs. To test this, PDE assays were first carried out on whole cell extracts of *P. aeruginosa* PAO1 (Figure 4.7a). A significant increase in PDE activity was observed in the presence of NO as compared to the untreated control group ($p < 0.0001$). Untreated samples had a rate of change of $0.0887 \pm 0.0043 \text{ h}^{-1}$ whereas 200 μM and 500 μM of S150 treated samples had a rate of change of $0.134 \pm 0.0055 \text{ h}^{-1}$ and $0.136 \pm 0.0056 \text{ h}^{-1}$ respectively. While treatment with 500 μM S150 yielded a slightly higher rate of change as compared to treatment with 200 μM S150, the difference was not significant ($p = 0.777$). Thus, exposure of the cell lysate to NO, enhanced the PDE activity relative to the untreated controls.

PDE assays were then carried out using the purified proteins of B2, R7, D5 and D8 to evaluate for any change in activity upon NO addition. At least 32 $\mu\text{g/mL}$ of proteins were used, based on the signal response curves shown above, which were within the linear range of activity and were well above the background absorbance. In contrast to the cell extracts, PDE activity was similar for these proteins in the presence and absence of 200 μM of S150 (Figure 4.6). B2 showed an activity of $0.0298 \pm 0.00087 \text{ h}^{-1}$ and $0.0294 \pm 0.00084 \text{ h}^{-1}$ in the absence and presence of S150 ($p = 0.786$). For R7, activity with or without NO treatment was at 0.0288 ± 0.00042 and 0.0304 ± 0.00086 respectively ($p = 0.127$). D5 had a rate of change at 0.00375 ± 0.00063 in the absence of NO treatment and 0.00420 ± 0.00060 in the presence of NO treatment ($p = 0.617$). Lastly, D8 had a rate of change of 0.0302 ± 0.00078 without NO treatment and an activity of 0.0289 ± 0.00040 with NO treatment ($p = 0.188$). Thus, none of the proteins or the subdomains tested, appeared to be responsible for the NO induced PDE activity observed for the cell lysates.

Figure 4.7 – PDE activity with NO treatment. PDE activity of *P. aeruginosa* cell extract (a), B2 (b), R7 (c), D5 (d) and D8 (e) in the presence or absence of 200 μ M S150 treatment over 3 h. Data represent values from at least two replicates whereas error bars represent the standard deviation of the mean.

4.3.4 Heme binding of RbdA

The presence of a heme group accounts for NO binding and sensing in PAS domains. Based on sequence alignment with the FixL PAS domain, the RbdA PAS domain was proposed to bind heme (An *et al.* 2010). In support of this, RbdA influences growth and biofilm formation under different aeration conditions, suggesting its role in sensing diatomic gases via a heme group (An *et al.* 2010). However, the purified RbdA protein was not observed to have a reddish hue typical of heme-binding proteins. Coupled with the observation that the enzymatic activity of RbdA was not modulated with NO addition, it was of interest to determine if RbdA could coordinate heme in its PAS domain. Sequence alignment of a predicted structure of RbdA PAS domain and FixL PAS domain using i-TASSER indicated that the PAS domain of RbdA is most structurally similar to 1d06A and 1y28A, both of which represent solved crystal structures of the FixL PAS domain, with TM-scores of 0.842 and 0.840 respectively (Appendix E). Protein-ligand prediction by COACH indicated that the most likely ligand of RbdA is heme, with the C-score at 0.55, where a score closer to 1 indicates a more reliable prediction. However, structural alignment of the modelled RbdA PAS domain with that of 1dp6 indicated that the ligand binding site of the modelled RbdA PAS domain is unable to accommodate the heme ligand, due to steric hindrance from residues such as Phe54, Leu68, Ile72 and Val113 surrounding the ligand binding site (Appendix E). In addition, a glycine group is present in place of histidine at the fifth coordination site of iron. This residue is conserved and essential for the activity of the heme prosthetic group, and usually occupied by histidine, cysteine or tyrosine. To directly test whether RbdA could bind heme, purified R7 and R12 were evaluated for heme binding using UV-Vis spectrometry. Purified proteins and cell pellets of R7 and R12 overexpressed in the presence of 5 μ M hemin did not display any reddish-brown tint characteristic of expression of heme-binding proteins (data not shown). The absorbance spectrum of heme binding proteins generally contain one distinct peak, known as a Soret peak, in the blue wavelength region, and two smaller

peaks between 530 nm to 630 nm. R7 did not display any heme binding, reflected in the lack of a Soret band at ~ 400 nm. R12, with only a PAS domain, showed a slight peak at ~ 412 nm, although the peak height was not proportional to the concentration of protein used (Figure 4.7), indicating that the signal may be noise rather than a true indication of the presence of heme.

Figure 4.8 – Heme binding in RbdA. Structure and heme binding of the FixL PAS domain (1dp6) (a) and structural alignment of the RbdA PAS domain generated by i-TASSER to 1dp6 using pymol (b). Representative UV-Vis spectrum of 0 – 2.5 μM of RbdA R7 (c) and 0 – 10 μM of R12 (d).

4.3.5 RNA sequencing

As DipA and RbdA were not found to act as direct sensors of NO, RNA-seq was carried out to identify other potential proteins that may play a role in NO induced dispersal. RNA samples were collected from 4 treatment groups, namely planktonic cells before NO treatment, biofilm cells before NO treatment, dispersed cells (i.e. NO treated planktonic cells) and NO-treated biofilms (i.e.

biofilms remaining following NO treatment). While genes related to iron regulation, dispersal (e.g. *bdlA*), virulence and regulation (i.e. sRNA) were found to be differentially regulated, the focus was on genes coding for DGCs or PDEs. This is because NO-induced dispersal is mediated through cdGMP, which is in turn regulated by the activities of both DGCs and PDEs. Recent studies had also indicated that a transient increase in cdGMP levels may precede a drop in intracellular cdGMP levels during dispersal, suggesting that dispersal signals may be first sensed and detected by DGCs, with PDEs functioning at a later point of the dispersal pathway (Basu Roy and Sauer 2014, Nair *et al.* 2017).

Figure 4.9 – Gene expression levels of PDEs and DGCs. Log₂ fold change of gene expression of genes related to regulation of cdGMP turnover with respected to untreated planktonic cells (a) and untreated biofilm samples (b) upon NO treatment.

RNA sequencing indicated that several genes related to cdGMP turnover, i.e. proteins with GGDEF domains or EAL domains, were differentially expressed ($\log_2$ fold change > 1 ; $p < 0.05$), as compared to expression levels in untreated planktonic or biofilm cells, upon NO treatment (Figure 4.9). Most genes were upregulated, to varying extents in the biofilms or in NO treated samples, except for PA1433, which was downregulated in NO treated samples but upregulated in biofilms, and PA2072, which was downregulated in biofilms (Figure 4.9a). The extent of gene expression differed between treatment groups, with *rbdA*, PA3177,

and *dipA* upregulated to a higher extent in the NO treated biofilm (Log₂ fold change at 1.414, 1.56, 1.09 respectively) as compared to NO dispersed planktonic cells (Log₂ fold change at 0.54, -0.2, 0.05 respectively), while *PA0575*, *PA2567*, *nbdA* and *morA* were upregulated to similar extents in both NO treated planktonic and biofilm samples (Figure 4.8a). *siaD*, which has only DGC activity, was also significantly downregulated in NO treated biofilms (-2.62 log₂ fold) in NO treated biofilms as compared to untreated biofilms (Figure 4.8b). In all, upon NO treatment, DGCs were downregulated while PDEs were upregulated, and genes implicated in NO induced dispersal, *nbdA*, *dipA* and *rbdA*, were upregulated to different extents in NO -treated planktonic and biofilm samples.

4.3.6 Selection of transposon mutants for screening

As neither RbdA nor DipA appeared to act as direct sensors of NO, other possible targets that may be involved in NO induced dispersal, such as genes involved in NO detoxification, respiration or modulation of cdGMP levels, were investigated to better understand the mechanisms of NO induced dispersal.

Transposon mutants in genes coding for DGCs and PDEs were first screened as these genes are known to be involved in biofilm formation and dispersal processes (Kulesekara *et al.* 2006, Valentini and Filloux 2016). In addition, transcriptomic analysis of *P. aeruginosa* treated with S150 showed that several such DGCs and PDEs encoding genes displayed changes in expression levels with NO exposure.

In addition to testing genes coding for PDEs and DGCs, transposon mutants of other genes involved in general biofilm dispersal, such as *bdlA*, a chemotaxis regulator essential for dispersal, were screened. Several genes, such as *dnr* and *anr*, were selected due to their roles in denitrification, for which *nirS* was important for both biofilm formation and NO induced dispersal (Barraud *et al.* 2006, Morgan *et al.* 2006, Trunk *et al.* 2010). In addition, transposon mutants of

global regulators such as *rpoN* and *rpoS* were evaluated due to their roles in general stress responses such as RNI stresses.

Despite screening of genes encoding DGCs, PDEs and genes related to other pathways of dispersal and NO response, no targets were identified. Another possibility might be that instead of a direct sensing mechanism, NO may act indirectly via inhibition of respiration. In support of this, factors that inhibit or affect respiration, such as the presence of cyanide or a reduction in oxygen levels, also induce dispersal (An *et al.* 2010). Further, NO has been shown to transiently affect respiration in *P. aeruginosa*, likely by inhibition of terminal respiration enzymes, electron transport chain and oxygen consumption (McCollister *et al.* 2011). Inhibition of respiration by NO does not affect NADH consumption, which is similarly observed with treatment with 100 – 200 μ M of S150 (McCollister *et al.* 2011). *P. aeruginosa* encodes several terminal oxidases with different affinities to oxygen, including the *aa*₃-type cytochrome *c* oxidase (*aa*₃), *cbb*₃-type cytochrome *c* oxidases (*cbb*₃-1 and *cbb*₃-2), *bo*₃-type quinol oxidase (Cyo) and bd family of cyanide-insensitive quinol oxidase (CIO) (Arai *et al.* 2014). Specifically, quinol oxidases, such as the *E. coli* cytochrome bd and bo' are known to bind and form complexes with NO, with NO interaction inhibiting oxidase activity (Butler *et al.* 1997, Hori *et al.* 1996, Stevanin *et al.* 2000). To evaluate the alternative hypothesis that NO induced dispersal may be sensed via terminal oxidases, several quadruple deletion mutants of the terminal oxidases were evaluated for defects in biofilm dispersal response to NO.

Lastly, genes related to chemotaxis and a newly identified heme-based NO-sensing pathway was evaluated. The former of which was selected as Aer-2 of the che2 chemotaxis-like system, contains a b-type heme in its PAS domain and is known to mediate chemotaxis in response to NO in *E. coli* (Garcia *et al.* 2017, Watts *et al.* 2011). The latter heme-based NO-binding and signal transduction pathway is reminiscent of H-NOX signalling pathways, in which the FIST domain of NosP functions similarly to H-NOX proteins as sensor domains, before transducing the signal to an effector protein such as a kinase in trans

(Hossain and Boon 2017). As no transposon mutant exists for the proposed sensor protein NosP (PA1975), mutants of the NosP associated histidine kinase (NahK, PA1976), which is also required for dispersal of *P. aeruginosa*, was tested for defects in dispersal response instead.

4.3.7 Transposon mutant screening

Mutants defective in NO induced dispersal were selected based on two criteria, a significant reduction in the extent of dispersal in response to NO treatment and mutants without a defect in biofilm formation. Of the tested mutants, only mutants of *rbdA* (Figure 4.9), *lapD*, *lapG* (Figure 4.10) and cytochrome c oxidase (QxAaS2) (Figure 4.12) displayed defects in dispersal relative to that of the wildtype. i.e. these mutants have a relative extent of dispersal of <1 as compared to the WT, and form sufficiently thick biofilms ($OD_{550} > 2$; black/ red colour scheme, figures 4.10 – 4.12a). Of these, only *rbdA* displayed a significant defect in NO induced dispersal ($p = 0.0001$, green bars in Figure 4.10b), while mutants of *lapD*, *lapG* and cytochrome c oxidase did not display any significant reduction in the extent of dispersal (Gray bars in Figure 4.11 and 4.12 respectively), suggesting that these genes may not be important in NO induced dispersal.

4.4 Discussion

It is now known that low concentrations of NO could act as a signal for biofilm dispersal (Barraud *et al.* 2006). Different studies had proposed various pathways through which NO treatment may be perceived by the cell and subsequently translate into a reduction in the intracellular cdGMP levels that leads to dispersal in *P. aeruginosa*. For example, NO was hypothesized to post translationally promote PDE activity (Barraud *et al.* 2009a). Alternatively, genes encoding PDEs may also be transcriptionally upregulated in response to NO treatment (Li *et al.* 2013a). In addition, multiple PDEs may be a part of a signalling cascade or

Figure 4.10 – Transposon mutant screening of mutants of PDEs/DGCs. Average biomass of transposon mutants of genes involved in cdGMP regulation in the absence or presence of NO treatment (a), and the relative extent of dispersal by the mutants in response to NO as compared to the MPAO1 WT (b). For (a), biofilm staining by CV of <1 is indicative of defects in biofilm formation and is represented as a green bar for samples that had not been treated with NO (NO-). For (b), bars represent data from three biological replicates while error bars represent the standard deviation of the mean. Values of 1 indicates similar extent of dispersal as compared to the WT, while values < 1 indicates a defect in dispersal to NO. Green bars are indicative of significant differences (p < 0.05) of mutants with defects in NO induced dispersal as compared to extent of dispersal by the WT, irrespective of defects in biofilm formation, based on ANOVA and multiple comparison with Dunnett's test.

Figure 4.11 – Transposon mutant screening of mutants of dispersal and NO metabolism. Average biomass of transposon mutants of genes involved in dispersal processes and NO metabolism, in the absence or presence of NO treatment (a), and relative extent of dispersal by mutants in response to NO as compared to the MPAO1 WT (b). Gray bars indicate mutants with non-significant extent of defects in dispersal to NO as compared to the MPAO1 WT strain.

Figure 4.12 – Transposon mutant screening of mutants of respiration and NO sensing. Average biomass of transposon mutants of genes involved in dispersal processes and NO metabolism, in the absence or presence of NO treatment (a), and the relative extent of dispersal by the mutants in response to NO as compared to the MPAO1 WT (b). For (b), gray bars indicate mutants with non-significant extent of defects in dispersal, while green bars are indicative of significant differences ($p < 0.05$) of mutants with defects in NO induced dispersal as compared to extent of dispersal by the MPAO1 WT strain. Bars represent data from three biological replicates while error bars represent the standard deviation of the mean.

pathway leading to dispersal (Basu Roy and Sauer 2014). Working under the hypothesis that NO post-translationally promotes PDE activity, and that the protein involved should be an active PDE, known to play a role in NO induced dispersal, with an NO sensing domain, two proteins, RbdA and DipA, were selected, purified and evaluated *in vitro* for changes in PDE activity upon NO treatment. The two proteins were not found to interact with NO directly. To further elucidate the mechanisms of NO-induced dispersal, genes involved in NO detoxification and dispersal pathways were screened. However, no new targets for NO-induced dispersal were identified.

4.4.1 NO-sensing by RbdA and DipA

P. aeruginosa cell extracts were first evaluated for changes in activity in response to NO treatment. Consistent with the literature, NO exposure resulted in an increase in PDE activity in the cell extracts (Barraud *et al.* 2009a). This change in PDE activity was similar for both 200 and 500 μ M of S150. This is in line with results of *in vitro* biofilm plate assays, where 200 μ M of S150 treatment was sufficient to disperse most of the biofilm, and there was no significant improvement in dispersal at higher concentrations.

In contrast to the *P. aeruginosa* cell extracts, no increase in PDE activities were observed for the purified BifA, DipA and RbdA proteins upon NO treatment, suggesting that DipA and RbdA do not act as direct sensors of NO. In support of this, the constructs RbdA R7 and DipA D5 used in this study did not appear to bind heme, which is an essential cofactor required for sensing of diatomic gases such as NO in PAS containing proteins (Gilles-Gonzalez *et al.* 1994, Gilles-Gonzalez and Gonzalez 2004, Rao *et al.* 2011). Structural alignment of the modelled PAS domain of RbdA to crystal structure of one of its closest homolog FixL (1Y28:A) also suggests that RbdA does not have a sufficiently large heme binding pocket in its PAS domain and does not have a histidine residue or imidazole group present at the fifth axial ligand position for heme coordination and oxygen binding activity (Barrick 1994, Zhang 2011). The same conclusions

had also been reached through homologous data base searches of the RbdA PAS domain, which returns target hits of PAS domains lacking associated heme groups (Liu *et al.* 2017). Crystallography studies of RbdA, which was carried out simultaneously using crystals of purified RbdA R7 (Figure 4.2), also confirmed a lack of a conserved histidine group (Liu *et al.* 2017). In addition, RbdA, together with BdlA and DipA, had been proposed to be part of a pathway leading to dispersal, indicating that the PAS domain may play other roles, such as the promotion of dimerization, instead of as a direct signal sensor (Basu Roy and Sauer 2014). Further, R12, consisting of only the PAS domain of RbdA, displayed a peak at 412 nm, corresponding to the Soret band of heme. RbdA was also proposed to bind heme based on sequence alignment between the PAS domain of RbdA and FixL, as well as the observation that RbdA plays a role in oxygen sensing (An *et al.* 2010). While such sequence alignment may not be reflective of structural homology and function, it should also be recognized that there may be considerable uncertainty in the predicted structure of RbdA that was used for structural alignment (He *et al.* 2017, Roy *et al.* 2009, Roy *et al.* 2010). In the case of R12, the signal observed at 412 nm was low and not proportional to the concentration of R12 used, suggesting that the signal may be due to contaminating proteins, or that any interaction with heme is weak. The discrepancy between the heme binding of R7 and R12 may also be due to construct design, as factors such as the selection or presence of affinity tags may influence the structure or activity of the protein (Chant *et al.* 2005, Rosano and Ceccarelli 2014, Sati *et al.* 2002). At present, it is evident that R7 does not bind heme, which may in turn contribute to the lack of modulation of PDE activity in response to NO treatment.

4.4.2 Evaluation of potential targets involved in NO induced dispersal

Transcriptomic analysis of *P. aeruginosa* treated with S150 showed that several sDGCs and PDEs encoding genes displayed changes in expression levels with NO exposure. The transcriptomic results were consistent with the current

literature, as genes previously noted to be transcriptionally upregulated upon NO treatment, such as *nbdA* (Li *et al.* 2013a), were found to be upregulated in both biofilms and planktonic cells treated with NO. Genes involved in NO induced dispersal, such as *rbdA* and *dipA* (An *et al.* 2010, Roy *et al.* 2012), were also upregulated. The transcriptional upregulation of *rbdA* and *dipA* suggests that these proteins function downstream of the signal transduction pathway. Downregulation of *siaD* (PA0169) expression in NO treated biofilms also suggest a role for DGCs in addition to PDEs, as SiaD is an active DGC with no PDE domains. Additionally, an increase in intracellular cdGMP levels prior to dispersal events also suggest that dispersal signals may be first sensed and detected by DGCs, with PDEs functioning at a later point of the dispersal pathway (Basu Roy and Sauer 2014, Nair *et al.* 2017). Surprisingly, no mutants in the Tn library other than *rbdA*, displayed defects in biofilm dispersal. *P. aeruginosa* encodes for several PDEs and DGCs (Kulesekara *et al.* 2006, Valentini and Filloux 2016). While the current mechanism of NO induced dispersal is unknown, it is possible that there is genetic redundancy of these genes (Kulesekara *et al.* 2006). Unexpectedly, both NbdA and DipA mutants also dispersed upon NO exposure in the system used, which contrasts with previous work where single deletion mutants in these genes were defective for NO-induced dispersal (Li *et al.* 2013a). Previous studies were carried out in continuous flow systems, where biofilms could reach maturation. As compared to the system of biofilm formation used in this study, the two systems also differ in shear and oxygenation. In the batch system used in this study, biofilms experience a higher shear as the plates were subjected to a high speed of shaking to promote oxygenation and biofilm formation. In contrast, biofilms in flow cell system used in the studies were developed under low, laminar flow conditions and anaerobic zones and gradients may exist within more mature biofilms (Stoodley *et al.* 2002, Yoon *et al.* 2002). Biofilms at different growth stages are also known to have a vastly different proteome and phenotype as compared to planktonic and immature biofilms (Stoodley *et al.* 2002, Thurlow *et al.* 2011). In

addition, certain genes, such as MucR, have different activities dependent on the growth phase of the bacteria, promoting biofilm formation via its DGC activity in planktonic cells while promoting dispersal via its PDE activity in biofilm cells (Li *et al.* 2013a). Furthermore, the PDE, DipA, also referred to as Pch, is known to be localized to the poles of a cell during cell division and account for heterogeneity in cdGMP levels during cell division (Kulasekara *et al.* 2013). Taken together, it is possible that the involvement of PDEs and DGCs may differ based on the experimental conditions used. As such, transposon mutants of genes previously found to be defective in NO induced dispersal were not detected with this screening.

Besides genes coding for PDEs and DGCs, transposon mutants of other genes involved important for biofilm formation and NO induced dispersal, such as *bdlA*, *dnr*, *anr*, and *nirS* were evaluated (Barraud *et al.* 2006, Morgan *et al.* 2006, Trunk *et al.* 2010). Transposon mutants of global regulators involved in nitrosative stress responses, such as *rpoN* and *rpoS* were also evaluated.

BdlA is unlikely to be a sensor of NO signal due to its role in dispersal in response multiple signal cues, such as succinate, glutamate and NO, and is likely to function downstream of the NO sensor (Basu Roy and Sauer 2014, Morgan *et al.* 2006). However, it was again unexpected that no defects in dispersal was observed with the loss of function of this gene, where deletion of the gene reduced dispersal had been shown to abolish dispersal, to NO or otherwise (Barraud *et al.* 2006, Morgan *et al.* 2006). The activity of BdlA requires activation by several proteases, DGCs and interactions with specific PDEs (Basu Roy and Sauer 2014, Petrova and Sauer 2012a, Petrova *et al.* 2015). Such specificity in regulation suggest that *bdlA* may be active or required for dispersal only under certain conditions, such as in biofilms grown in flow systems, as observed in previous studies (Barraud *et al.* 2006, Morgan *et al.* 2006). Similarly, *dnr*, a primary NO sensor of *P. aeruginosa*, displayed a low affinity to NO (micromolar range), which is inconsistent with the nanomolar concentrations of NO required for dispersal (Giardina *et al.* 2008). Dnr is hence unlikely to function

as a direct sensor of NO during dispersal. The study suggests that Dnr does not play a role in NO induced dispersal under the conditions tested, likely because Dnr mainly functions under low-oxygen conditions (Schreiber *et al.* 2007). While mutants of *lapD* and *lapG* showed defects in dispersal, the effects were not significant as compared to the WT controls, likely due to variations in extent of defects between different biological replicates. This defect in dispersal is not surprising for the mutant of *lapG*, for which the encoded LapG is involved in the cleavage of LapA adhesion for biofilm dispersal downstream of cdGMP regulation (Newell *et al.* 2009, Rybtke *et al.* 2015). However, a mutation of *lapD* should result in hyper-dispersal, as LapD binds to LapG at high cdGMP concentrations to prevent activity of LapG (Newell *et al.* 2009). This suggests that LapD may play additional roles leading to dispersal via LapG cleavage of LapA (or CdrA in *P. aeruginosa*). Irrespective of the role of LapD, the contribution of LapG to NO induced dispersal is likely non-specific and rather is the result of a general defect in dispersal.

Working on the hypothesis that NO may act via a newly discovered heme based NO-sensing pathway, modulation of motility related genes or indirectly via inhibition of respiration, mutants of *nahK*, *aer-2* and the *P. aeruginosa* respiratory chain were screened. As with previous attempts, these genes were not found to be involved in NO induced dispersal. For mutants of NahK, dispersal by NO was carried out in batch systems, not unlike the model used in this study, with the distinction that NO treatment was carried out overnight in 96 well plates incubated statically (Hossain and Boon 2017). NO treatment was administered prior to biofilm formation, and differences in the experimental set-up may have accounted for a lack of defects in NO induced dispersal in our set-up. While Aer-2 can respond to diatomic gases such as carbon monoxide, oxygen and nitric oxide, its endogenous ligand is likely to be oxygen (Garcia *et al.* 2017). In addition, Aer-2 mediated chemo or aero taxis had not been observed and the Che2 system that Aer-2 belongs to does not bind the flagella motor protein (Garcia *et*

al. 2017). Together, this suggest that the gene likely does not mediate motility and dispersal in response to NO but play a role besides chemo- or aerotaxis.

Lastly, each of the terminal respiratory oxidases have different affinities to oxygen and are upregulated under different conditions (Arai 2011a). Of which, the *cbb₃* oxidases (*ccb₃-1* and *ccb₃-2*) are known to have a very high affinity for oxygen and is expressed constitutively at high levels or induced under low oxygen conditions (Comolli and Donohue 2004, Kawakami *et al.* 2010). Hence, under the high oxygen conditions tested, the *cbb₃* oxidases may not play a significant role or would have preferentially bind its endogenous oxygen ligand. The *aa₃* type terminal oxidase is encoded by the *cox* gene and has the highest ability to generate a proton gradient. Consequently, the gene is expressed at low levels even with under high oxygen levels, likely to prevent imbalances in redox homeostasis (Arai 2011a, Kawakami *et al.* 2010). NO may hence have limited influence on *aa₃* due to its low expression levels. *bo₃* oxidases, encoded by *cyo*, is similarly expressed in low levels. The gene is regulated by the transcriptional regulator, Fur, and is induced under low iron conditions or in the presence of NO (Kawakami *et al.* 2010, Ochsner *et al.* 2002). Despite being regulated by NO, mutants expressing only *cyo* did not display any differences in dispersal with NO addition (Figure 4.12). This could be because the role of Cyo in nitrosative stress resistance is unclear, and the effects exerted by NO may simply be due to inactivation of Fur by NO, and not a direct effect of NO on the enzyme (Arai 2011a, D'Autreaux *et al.* 2002). The last terminal oxidase encoded by *cio*, functions as an electron sink under cyanogenic conditions, as *P. aeruginosa* produces hydrogen cyanide under low oxygen conditions (Cunningham and Williams 1995, Cunningham *et al.* 1997). The gene is induced in the presence of respiratory chain inhibitors, including the NO donor sodium nitroprusside (SNP) and is regulated by genes involved in stress response or denitrification processes, such as RoxSR, RpoS and Anr. Addition of NO to a mutant expressing only *cio* also had no effect on NO-induced dispersal, likely because the effect exerted by SNP is not due to NO per se, but due to the presence of CN- groups of SNP.

Further, regulation of *cio* by ANR is not related to NO production, but oxygen levels as the regulator prevents expression of the gene when it is not required (Arai 2011a).

4.5 Conclusions and future directions

It is now known that several systems contribute to NO induced dispersal. The NosP/NarK phosphorelay signal transduction system, the BdlA/DipA/RbdA cascade leading to modulation of intracellular cdGMP levels, downstream transcriptional upregulation of PDE genes such as *nbdA* and post-translational activation of PDE activity. Previous studies indicated that single deletion mutants were sufficient to abolish dispersal in response to NO treatment, as the proteins are part of a pathway. However, based on the screen of transposon mutants, with the exception of *rbdA*, none of the previously identified mutants were defective in NO induced dispersal. Functional redundancy and differences in the experimental setup used may have contributed to the differences observed as different genes may be involved depending on the growth conditions, and oxygen levels may influence reactions with NO. Interestingly, RbdA has also been implicated in increased biofilm formation under anaerobic conditions (An *et al.* 2010). Hence, RbdA either promotes biofilm formation in the presence of NO generated through denitrification under anaerobic conditions or dispersal in the presence of exogenously added NO under aerobic conditions. At present, mutants of DipA, RbdA, MucR and NbdA had also been shown to be defective in NO-induced dispersal when evaluated using mature biofilms, in which steep nutrient and oxygen gradients are present. In this study, however, NO was added to immature biofilms formed under high oxygenation conditions. It would hence be of interest to determine how each gene may contribute to NO-induce dispersal through exogenous NO addition of biofilms developed under various oxygenation conditions and stages of biofilm development. The latter of which known to display distinct physiology and protein expression (Sauer *et al.* 2002).

In addition, mutants with multiple deletions could be generated and their response to NO-induced dispersal compared to that of single mutants. *P. aeruginosa* codes for > 30 PDEs. Hence, a good starting point would be to target genes involved in NO-induced dispersal, such as DipA, RbdA, MucR and NbdA. For example, DipA has been known to associate with the chemotaxis regulator, BdlA to promote biofilm dispersal in response to glutamate (Roy *et al.* 2012). At the same time, DipA is also known to associate with CheA to facilitate its localization at the poles during cell division and requires CheA for its activity (Kulasekara *et al.* 2013). During cell division then, DipA is thought to promote diversity and variation in chemotactic responses, hence promoting survival of the population (Kulasekara *et al.* 2013). However, there had been no clear link between CheA, the localization of DipA and dispersal events, such as NO-induced dispersal, which typically coincide with the generation of flagellated populations and cell lysis, rather than cell division and growth (Webb *et al.* 2003). Evaluation of NO-induced dispersal of double deletion mutants of e.g. *dipA* and *nbdA*, or in other combinations, could also provide insights into their regulation or redundancy, as these two pathways had been investigated separately, but not known to be part of the same signalling pathway.

5. Summary and general discussion

5.1 Summary

In this thesis, the application of novel NO donors as a biofilm control strategy was investigated. The study provided insights into the use of extended NO release and hybrid-NO prodrug compounds in biofilm control. In addition, the mechanisms of NO sensing and dispersal were examined.

Furoxans were found to induce dispersal, promoted growth and/or inhibit pyoverdine production via independent mechanisms, suggesting that they may be used as dispersal agents or for further development as pyoverdine inhibitors. Unlike NO donors with short half-lives, extended NO release compounds, such as stable cephalosporin-linked hybrid compounds, are advantageous for targeted NO delivery due to reduced diffusibility of reactive NO from the site of activation. The use of NO donors *in vivo* may be limited by their pharmacokinetics, such as the adsorption of the compounds onto the cornea in a mouse keratitis infection model. Furthermore, efficacy of NO donors in inducing biofilm dispersal may be reduced due to resistance mechanisms by the bacteria, such as NO detoxification by NO dioxygenases, like Fhp. Hence, while NO-based treatments, in combination with antimicrobial therapy, may offer a novel strategy for controlling biofilms, further studies are required to optimize conditions for the application of these compounds.

At present, the molecular mechanism of NO sensing and dispersal in *P. aeruginosa* biofilms remain poorly understood. The process involved cdGMP, although PDEs like RbdA and DipA were unlikely to be sensors of NO. Furthermore, genes involved in NO mediated dispersal were not found to be defective in the phenotype during our screening process, suggesting that there may be genetic or functional redundancies in the NO-induced dispersal response.

5.2 Reactivity, metabolism of and resistance to NO and its implications in application and dispersal

NO is a highly reactive compound that is involved in various physiological processes (Figure 6.1). Several factors, such as the flux and sustainability of flux (i.e. extended release of NO), influence the physiological response to NO (Schonafinger 1999, Thomas *et al.* 2006, Thomas *et al.* 2008a). In mammalian tumour development, for example, the sustainability of flux appears to play a more important role than the initial burst of NO from an NO donor, as a sustained release of $\geq 50 \mu\text{M}$ S150 ($t_{1/2} = 39 \text{ min}$, 37°C , $\text{pH} = 7$) resulted in the accumulation of the hypoxic inducible factor 1α (HIF- 1α), which is generally associated with tumour proliferation, while a rapid release of NO from DEA NONOate ($t_{1/2} = 2 \text{ min}$, 37°C , $\text{pH} = 7$) at an effective concentration of $2 \mu\text{M}$ for less than 1 hour had no effect (Thomas *et al.* 2004). In the case of NO-induced dispersal in *P. aeruginosa*, the flux of NO appears to play a more important role, as dispersal could be observed within short time frame of 15 min following exposure to NO donors, and a range of NO donors i.e. S150, DEA NONOate, Proli NONOate, with half-lives ranging from 2 s to 39 min, had been effective in inducing biofilm dispersal (Chapter 3). Furthermore, short exposure (1 h) to low concentrations ($100 - 500 \mu\text{M}$) of the extended NO releasing furoxans LL4212 and LL4216 did not result in dispersal, but the same compounds could disperse the biofilms when used at a higher concentration (3.6 mM) (Chapter 2). While the initial concentration of NO may be important for inducing biofilm dispersal, extended NO release compounds remain useful as studies had indicated a role of NO in preventing biofilm formation. In these cases, such compounds, may first serve as dispersing agents, then act to prevent further biofilm formation.

Figure 5.1 – Direct and indirect effects of NO that are dependent or independent of oxygen concentrations. Figure is adapted from (Thomas *et al.* 2006) and reproduced with permission from John Wiley and Sons.

Responses mediated by NO are dependent on the flux and sustainability of NO. In turn, these factors are influenced by oxygen, as NO reacts with oxygen to form other reactive nitrogen or oxygen species that may modify proteins (Figure 6.1). Under aerobic conditions, the consumption of NO is increased due to reactions with oxygen (Thomas *et al.* 2006, Thomas *et al.* 2008a). On the other hand, NO affects respiratory processes under anoxic or anaerobic conditions (Arai 2011b). As such, a balance between oxygen and NO levels is required for the regulation of metabolic and physiological processes. Indeed, computational and experimental studies had indicated that oscillations of NO and oxygen concentrations occur in *E. coli* and *P. aeruginosa* under certain oxygenation conditions, due to competition for oxygen between the NO dioxygenase Hmp of *E. coli* (functionally similar to Fhp of *P. aeruginosa*) and respiratory cytochromes, and inhibitory effects of available oxygen on the NO reductase (NOR), NOV, of *E. coli* (homologous to NOR of *P. aeruginosa*) (Robinson and Brynildsen 2016).

The importance of the bioavailability of NO due to generation or consumption and loss in the presence of oxygen, is again exemplified in studies with HIF-1 α , where accumulation of HIF-1 α , with a steady-state of NO release, only occurred under low cell density, as NO is readily consumed at high cell densities. Furthermore, with higher concentrations of the NO donor S150 used, NO predominantly promoted p53 phosphorylation, associated with apoptosis, while lower concentrations of S150 promoted HIF-1 α accumulation, associated with tumour growth and differentiation (Hofseth *et al.* 2003, Thomas *et al.* 2004). As such, to study the role of these proteins in tumour development, oxygen gradients within a tumour must be considered. Such gradients, are similarly important in the context of biofilms and the use of NO for biofilm control, where NO production, and subsequently, dispersal, occurs after establishment of an oxygen gradient and transition from aerobic to anaerobic respiration in oxygen-poor regions, with NO displaying comparable biphasic effects of promoting biofilm formation at higher NO concentrations (>25 mM SNP, equivalent to ~25 μ M NO) (Barraud *et al.* 2006).

Although endogenous NO production may be dependent on oxygen levels in biofilms, exogenous additions of NO continue to mediate dispersal in the presence of oxygen, as biofilms were able to disperse in the oxygenated microplate experimental setup used (Chapters 2 – 4). Exogenously added NO was unable to disperse biofilms formed under anaerobic conditions in the presence of nitrate (data not shown), likely due to NO detoxification by NOR. Such processes of NO detoxification, by NOR or Fhp, may contribute to the resistance of *P. aeruginosa* biofilms to further dispersal by NO (Chapter 3). Previous studies of NO consumption under low oxygen conditions indicated that, following NO loss due to escape to air (~60%), detoxification by bacteria NO consumption account for the majority (~36%) of NO consumption (Robinson and Brynildsen 2016). Furthermore, modelling of oxygen dynamics in CF sputum had shown that anoxic zones vary depending on bacterial load and mucus thickness, and significant portions of CF mucus volumes may be anoxic in nature

(Cowley *et al.* 2015). Since NO detoxification enzymes such as NO dioxygenases and NO reductases are common amongst bacterial species, it is likely that these responses are conserved, and resistance mechanisms and activity of these proteins under varying oxygen conditions must be considered when administering NO donor compounds for biofilm control.

The interplay between oxygen and NO reactions, metabolism and detoxification may influence biofilm dispersal under different environmental conditions. However, it does not account for dispersal by NO of mutants of genes known to be involved in the process (Chapter 4). While higher oxygenation in the experimental system used may enable reactions with oxygen and consumption of NO, dispersal by the wildtype strain indicated that bioavailability of NO is not a factor affecting the process. In this case, it is hence likely that dispersal is differentially regulated depending on the growth conditions. Further studies would be required to determine how differences in growth conditions may affect the dispersal response, or if the effects of NO in this process are direct (via metal nitrosylation), or indirect (via reactions with protein side chains).

In summary, the biochemistry of NO complex and is influenced mainly by NO and oxygen concentration, either directly through chemical reactions, or indirectly through NO detoxification and metabolism under different oxygenation conditions. These reactions, along with NO synthesis or release from NO donors, influences the flux and sustainability of NO release, which in turn affects NO response, and may account for variability in dispersal when administered as biofilm control agents. As such, they are important factors to consider when assessing new NO based drugs. However, it should be noted that NO and oxygen levels are not the sole factors influencing the dispersal response and further understanding of the mechanism of NO dispersal is required to identify other determinants at play. Future studies of NO application could hence be carried out over a range of oxygen conditions to better address variability in the dynamics of biofilm dispersal and in response to NO treatment.

5.3 Translatability – Opportunities, risks and challenges of the use of NO-donors for biofilm dispersal

In the above section, the reactivity of NO, its effects on the effective NO concentration sensed by cells and its physiological outcome was discussed. Similarly, the opportunities, risks and challenges of the use of NO in biofilm treatment can be traced back to the same factors, its reactivity, ubiquitous nature and role as a signalling molecule. As established, effects modulated by NO are concentration dependent, which in turn is dependent on its reactivity (Figure 5.2). For example, due to the reactivity of NO, it must be generated near the site of biofilm infection. At present, this can be achieved using bacteria activated prodrugs (chapter 3), and the challenges involving the development of said compounds had been discussed in Chapter 3.

Figure 5.2 – Outcomes of NO treatment at different concentrations. The reactivity of NO accounts for the challenges in its use, due to the ind

uction of undesirable or non-specific effects, but also present opportunities through which different processes may be modulated at the same time. E.g. Antimicrobial effect with improved host antimicrobial defence.

Another challenge lies in the effective NO concentration suitable for inducing biofilm dispersal. At low NO concentrations, NO treatment induces biofilm dispersal. One of the challenges lies in the fact this concentration is both bacteria and biofilm dependent. For example, in one study, low concentrations of NO (~500 nM SNP) induced biofilm dispersal in *P. aeruginosa* grown on glass surfaces whereas higher concentrations (μM – mM) increases biofilm formation (Barraud *et al.* 2006). However, in a separate study, 500 μM of SNP was able to disperse *P. aeruginosa* biofilms grown on silicone tubings (Roy *et al.* 2012). Similarly, 100 – 500 nM of SNP could disperse *B. licheniformis* biofilms formed on glass slides with > 90% reduction in coverage while 10 μM of SNP reduced coverage of *S. epidermidis* biofilm formed under the same conditions by only ~ 58% (Barraud *et al.* 2009b). A more detailed summary of the use of NO can be found in Appendix A.

At concentrations that are too low to induce dispersal, NO may also induce adaptive responses. For example, a recent study had shown that the addition of non-dispersing concentration of NO resulted in the upregulation of *fhp*. Fhp is involved in the detoxification of NO. Subsequently, the biofilms could not be dispersed using a higher concentration of NO, as compared to the control groups not previously treated with NO (Zhu *et al.* 2017). Such adaptive responses hence pose a problem for repeated treatment with NO, where NO may promote biofilm formation instead of dispersal.

Lastly, at sufficiently high concentrations, NO is toxic and will kill bacteria within biofilms. However, at these high concentrations NO may also cause damage to the host. High levels of NO were reported to inhibit macrophage iNOS expression and inflammation, thereby resulting in reduced healing and loss of macrophage activity at the site of infection (Peng *et al.* 1998). Furthermore, high levels of NO may reduce lymphocyte proliferation and inhibit leucocyte activation (Lloyd-Jones and Bloch 1996, Moilanen and Vapaatalo 1995). Despite challenges in establishing a suitable NO concentration for its application, reduced

NOS activity has also been associated with poor antimicrobial defence in CF lungs and inflammation disorders (Chmiel and Davis 2003, Michl *et al.* 2013, Poschet *et al.* 2006). NO has also been implicated in wound healing processes (Blakytyn and Jude 2006). The use of NO as a treatment option, in these cases, would provide a mean to address different issues related to the establishment of chronic biofilm infections, at the same time. For the former, supplementation of NO could then target both *P. aeruginosa* infections in CF patients, and inflammation dysregulation.

Due to the multifaceted effects of NO, more studies must be carried out to ensure effectiveness of NO towards its targeted outcome, while minimising adaptive biofilm responses and negative effects on host cells. At present, the use of NO for biofilm dispersal had been established *in vitro*. Hence, there is greater opportunities in its application in non-clinical settings, such as the dispersal of biofilms formed on surfaces or industrial membranes (Barnes *et al.* 2015)

Appendix A – List of studies on NO and biofilm inhibition and dispersal

Compound	Concentration	Bacteria/ Sample	Effects of NO	References
Gaseous NO (gNO)				
gNO applied directly to wound site	200 ppm	<i>Staphylococcus aureus</i> on rabbit <i>in vivo</i> wound infection models	Antimicrobial effects, promotion of wound healing	(Ghaffari <i>et al.</i> 2007)
		Nonhealing chronic venous ulcer in a 55-year-old male		(Miller <i>et al.</i> 2004)
gNO released via wound dressing	~200 ppm	<i>Acinetobacter baumannii</i> , <i>MRSA</i> , and <i>P. aeruginosa</i> biofilms on polyvinyl plates	Antimicrobial effects	(Sulemankhil <i>et al.</i> 2012)
Inhaled gNO	160 ppm	<i>P. aeruginosa</i> pneumonia model in rats	Antimicrobial effects without influence on neutrophil myeloperoxidase function, methemoglobin % or plasma nitrite/nitrate levels	

Inhaled gNO	40 ppm	Intratracheal instillation of <i>P. aeruginosa</i>	Antibacterial effect with reduced pulmonary leukocyte infiltration <i>in vivo</i> .	(Webert <i>et al.</i> 2000)
Inhaled gNO with exposure to oxygen (FIO ₂ ~100%)	10 ppm	Pneumonia induced via alveolar instillation of live <i>P. aeruginosa</i> in Male Sprague-Dawley rats in rats.	Bactericidal effect against <i>P. aeruginosa</i> without modification of alveolar neutrophil functions.	(Jean <i>et al.</i> 2002)
Inhaled gNO	5 ppm	<i>P. aeruginosa</i> biofilms of CF patients, codelivery with IV tobramycin and ceftazidime	Reduction of <i>P. aeruginosa</i> biofilms and improved lung function	(Katrina <i>et al.</i> 2014)
NO donor compounds				
SNP	25 to 500 nM	<i>P. aeruginosa</i> biofilm developed on glass surface in flow system	Reduction of <i>P. aeruginosa</i> biofilm surface coverage via induction of biofilm dispersal; Promotion of biofilm eradication when used in conjunction with other antimicrobials	(Barraud <i>et al.</i> 2006)
	500 µM	<i>P. aeruginosa</i> biofilm developed on continuous flow tube reactor system composed of Masterflex size 13 silicone tubing	Induction of biofilm dispersal	(Li <i>et al.</i> 2013a, Roy <i>et al.</i> 2012)

	25 - 500 nM	<i>Serratia marcescens</i> biofilm on unplasticized polyvinyl chloride slides	Up to 60% reduction in biofilm coverage	(Barraud <i>et al.</i> 2009b)
	500 nM	<i>Vibrio cholerae</i> biofilm on glass slides	73% reduction in surface coverage. Enhance tetracycline activity against <i>V. cholerae</i> biofilm when used in conjunction with SNP	
	1 - 10 μ M	<i>Fusobacterium nucleatum</i> biofilm	Inhibition of biofilm formation. 48 – 55% reduction in biofilm surface coverage	
	100 - 500 nM	<i>Bacillus licheniformis</i> biofilm on glass slides	> 90% reduction in biofilm surface coverage	
	10 μ M	<i>Staphylococcus epidermidis</i> biofilm on glass slides	58 % reduction in surface coverage	
	25 – 100 nM	<i>Candida albicans</i> biofilm on polystyrene plates	Up to 61 % reduction in biofilm formation	
SNP	500 nM	3-month-old multispecies biofilm developed on unplasticized polyvinyl chloride	~47 % biofilm dispersal. Increased sensitivity to chlorine (HOCl) treatments	(Barraud <i>et al.</i> 2009b)

		coupons in recycled water network		
	100 nM	3-month-old multispecies biofilm developed on reverse osmosis membrane connected in line with a full-scale water filtration system	~30% reduction in biofilm viability. Combined exposure to both SNP and chlorine resulted in 94 % reduction in biofilms	
Diazenium diolates (NONOates)				
Proli NONOate	20 – 500 nM	3-month-old multispecies biofilm developed on reverse osmosis membrane connected in line with a full-scale water filtration system	Up to 41 % reduction in biofilm. Increased efficacy of chlorine at removing biofilm cells	(Barraud <i>et al.</i> 2009b)
	40 µM	RO modules inoculated with <i>P. aeruginosa</i> or mixed bacterial community isolated from industrial MBR and RO membranes	Reduction in biofilm surface coverage and thickness of biofilm on RO membrane accompanied by a reduction in polysaccharides proteins, and microbial cells	(Barnes <i>et al.</i> 2015)
	1 – 80 µmol l ⁻¹	<i>P. aeruginosa</i> biofilm	Maximal dispersal of ~ 30 %	(Barnes <i>et al.</i> 2013)

MAHMA NONOate	1 – 80 $\mu\text{mol l}^{-1}$	<i>P. aeruginosa</i> biofilm	Maximal dispersal of ~ 40 %. Multiple dosing increased the extent of reduction of biovolume. Induction of dispersal from the biofilm, with secondary effect of inhibition of biofilm growth	(Barnes <i>et al.</i> 2013)
MAHMA NONOate	10 pM – 10 μM	<i>Salmonella</i> 14028, <i>E. coli</i> O157:H7 or <i>Salmonella</i> cocktail biofilm developed at 37°C on polypropylene or polystyrene surfaces for 24 h followed by treatment at 22°C or 4°C for 6 and 24 h	Varying effects dependent on temperature, strain and surface, with MAHMA NONOate being a strong dispersant of the <i>Salmonella</i> biofilms on polystyrene. Effects were inverse, with pM treatment showing higher extent of dispersal	(Marvasi <i>et al.</i> 2014)
Spermine NONOate				<i>*Treatment carried out in PBS. Hence, dispersal may be due to carbon starvation, and inverse effect of treatment may be due to polyamine based NONOates functioning as a nutrient source allowing for biofilm maintenance. This is not explored in the paper, as no CPTIO controls or polyamine controls were included.</i>
DEA NONOate			Dose dependent inverse effect, with higher concentration of diethylamine NONOate resulting in lower extent of dispersal following treatment	
Other soluble NO donors				

SNAP	1 μ M	<i>V. cholerae</i> biofilm on glass slides	29% reduction in surface coverage	(Barraud <i>et al.</i> 2009b)
GSNO			34% reduction in surface coverage	
SNAP	10 pM – 10 μ M	<i>Salmonella enterica</i> , <i>E. coli</i> and <i>Listeria</i> biofilms developed on 96-well polypropylene or polystyrene plates at 37°C for 18 h, followed by treatment at 4°C for 6 h	Up to 30% biofilm dispersal	(Marvasi <i>et al.</i> 2016) * Similar comments as for (Marvasi <i>et al.</i> 2014). Further, molsidomine activation is unclear, as mentioned in text.
Molsidomine	10 nM – 10 μ M	<i>Pectobacterium carotovorum</i> biofilms developed on 96-well polypropylene or polystyrene plates	Up to 30 % biofilm dispersal, with synergistic effects when used in combination with sanitizers	However, NO mediated <i>ygaD</i> , <i>mltB</i> , <i>srlR</i> , and <i>gutQ</i> genes detected to be upregulated, suggesting NO is involved.
Hybrid compounds				
Fimbricide–Nitric Oxide Donor Hybrids	3.2 – 266 μ M	<i>P. aeruginosa</i> biofilms	QS repressing activities. Inhibition of biofilm formation dependent on NO donor group	(Kutty <i>et al.</i> 2013)
Cephalosporin linked diazeniumdiolates	0 – 100 μ M	<i>P. aeruginosa</i> biofilms pretreated with 0.5 μ g/mL imipenem	Up to 70 % decrease in biofilm, potentiation of tobramycin and ciprofloxacin treatment	(Barraud <i>et al.</i> 2012)

	10 nM – 100 μ M	<i>Haemophilus influenzae</i> (NTHi) <i>in vitro</i> biofilms developed on polystyrene surface	Lack of biofilm dispersal, but No-mediated increased NTHi <i>in vitro</i> biofilm susceptibility toward azithromycin treatment when used in combination with the antibiotic	(Collins <i>et al.</i> 2017)
NO release from gel like or solid surfaces				
NO release from xerogel polymers coated with fibrinogen	NO flux 0 – 30 pmol/cm/s	<i>S. aureus</i> , <i>S. epidermidis</i> and <i>E. coli</i> adhesion on PVC-coated NO-releasing and control xerogels soaked in bacterial suspension for 90 min	NO flux on the fibrinogen coated polymer surface correlated strongly to reduction of <i>S. aureus</i> , <i>S. epidermidis</i> and <i>E. coli</i> adhesion, with higher effectiveness against <i>S. aureus</i> and <i>E. coli</i>	(Charville <i>et al.</i> 2008)
NONOate linked to gold nanoparticle via (P(OEGMA)-b-PVBC) copolymer	1 – 10 ppm	<i>P. aeruginosa</i> biofilm developed on polystyrene plates	Concentration dependent reduction in biofilm surface coverage (adhesion) and planktonic optical density	(Duong <i>et al.</i> 2014)

Appendix B

Dispersal of *P. aeruginosa* biofilm by the NONOate S150

Figure B-1– Structure of spermine (a) and spermine NONOate, S150 (b). Spermine has been included as a negative control in addition to samples with added NO scavenger cPTIO. The compound does contain the $-(NO-)-N=O$ moiety and does not release NO in solution.

Figure B-2 – Remaining biomass, as quantified by crystal violet staining, of samples treated with medium (control), 200 μ M spermine, 200 μ M S150 or 200 μ M S150 together with cPTIO. Bars represent data from triplicate wells and error bars represent the standard deviation of the mean. Data was analysed using one-way ANOVA with multiple comparison in Graphpad prism V7.02. ****, $p \leq 0.0001$.

Figure B-3 – CFU present in biofilm and planktonic samples following treatment with medium (control), 200 μ M spermine, 200 μ M S150 or 200 μ M S150 together with cPTIO. Bars represent data from triplicate wells and error bars represent the standard deviation of the mean. The height of the stacked bars, representing the total CFU from both planktonic and biofilm samples, is similar across samples, indicating that no cell death was induced through the addition of S150.

Figure B-4 – Biovolume of samples treated with medium (control), 200 μ M spermine, 200 μ M S150 or 200 μ M S150 together with cPTIO. Bars represent data from two samples with three image stacks and error bars represent the standard deviation of the mean. Data was analysed using one-way ANOVA with multiple comparison in Graphpad prism V7.02. **, $p \leq 0.01$; ***, $p \leq 0.001$.

Appendix C

Ongoing studies with cephalosporin-NO donor prodrugs designed to be activated by PBPs

Table C-1 – Structure of newly synthesized compounds

Compound	Structure	Cephalosporin	NONOate
UW11		Ceftazidime	AminoPip1
UW12		Ceftazidime	AminoPip2
UW13		Cefaloram	AminoPip1
UW14		Cefaloram	AminoPip2
UW15		Cefotaxime	AminoPip1
UW16		Cefotaxime	AminoPip2

Table C-2 - MIC of compounds

Compound/ Antibiotic	MIC ($\mu\text{g/mL}$)
UW11	4
UW12	8
UW13	128
UW14	128
UW15	32
UW16	32
Ceftazidime	16
Cefaloram	>128
Cefotaxime	128

Figure C-1 – Workflow of future plans with newly designed hybrid NO-donor compound

Appendix D

Transposon mutant strains used in study (Chapter 4)

Strain	Relevant genotype	Source
<i>Pseudomonas aeruginosa</i>		
MPAO1	WT	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW1288	PA0169-siaD-F06::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW1532	PA0290-B11::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3000	PA1107-roeA-A03::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3024	PA1120-C01::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW4247	PA1851-H02::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW5641	PA2771-E11::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW5818	PA2870-B06::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW6316	PA3177-G07::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW6632	PA3343-H12::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW8314	PA4332-sadC-B07::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW8424	PA4396-D06::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW9146	PA4843-gcbA-H02::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW10280	PA5487-C09::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW4748	PA2200-D01::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW7454	PA3825-G08::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW1520	PA0285-H01::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW5581	PA0575-D07::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)

MPAO1 tn-PW2570	PA0861-rbdA- A03::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3133	PA1181-D05::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3603	PA1433-B12::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW4044	PA1727-mucR- A06::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW4568	PA2072-B02::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW6568	PA3311-nbdA- G07::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW8372	PA4367-bifA- C04::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW8754	PA4601-morA- C02::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW9347	PA4959-fimX- A08::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW9425	PA5017-dipA- H11::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW9919	PA5295-H12::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW10194	PA5442- B07::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3784	PA1544-anr-E05::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW1966	PA0527-dnr- G02::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3587	PA1423-bdIA- F03::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW9303	PA4929-nicD- F02::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3603	PA1433-lapD- B12::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW3606	PA1434-lapG- D03::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)

MPAO1 tn-PW8507	PA4462-rpoN- H02::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW7152	PA3622-rpoS- H02::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW5460	PA2665-fhpR- F06::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW2216	PA0408-pilG- C07::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW2975	PA1094-fliD- B03::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW10274	PA5483-algB- D11::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW4441	PA1974-G07::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW4443	PA1976-E02::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW1299	PA0175-D08::ISphoA/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW1303	PA0176-aer2- E11::ISlacZ/hah	(Jacobs <i>et al.</i> 2003)
MPAO1 tn-PW1308	PA0175-D05::ISphoA/hah	(Jacobs <i>et al.</i> 2003)

Appendix E

RbdA i-Tasser and crystallization results

Figure E-1 – Sequence alignment RbdA PAS domain with top ten proteins hits from the Protein data bank (Pdb). Boxed residues (His) indicate location of conserved histidine for heme coordination.

Table E-1 – Sequence identity, coverage and normalized Z-score of top 10 hits based on sequence alignment in Figure E-1.

Rank	PDB Hit	Ident1*	Ident2*	Cov*	Norm Z-score*
1	user	0.26	0.28	0.86	10.00
2	1y28A	0.25	0.28	0.85	2.84
3	1drmA	0.27	0.27	0.87	2.48
4	1y28A	0.25	0.28	0.87	2.19
5	1dp6A	0.26	0.26	0.85	2.13
6	1ew0A	0.21	0.24	0.90	2.95
7	1ew0A	0.21	0.24	0.90	2.28
8	1ew0A	0.21	0.24	0.90	3.09
9	1ew0A	0.21	0.24	0.90	2.67
10	1ew0A	0.21	0.24	0.90	2.63

* Ident1 is the % sequence identity of the templates in the threading aligned region with the query sequence. Ident2 is the % sequence identity of the whole template chains with query sequence. Cov represents the coverage of the threading alignment and is equal to the number of aligned residues divided by the length of query protein. Norm. Z-score is the normalized Z-score of the threading alignments. Alignment with a Normalized Z-score >1 mean a good alignment and vice versa.

Top 5 predicted models by i-TASSER

Table E-2 – Predicted models and scores of predicted structures against native structures.

Model 1	Model 2	Model 3	Model 4	Model 5

Name	C-score*	Exp. score*	TM-Exp. RMSD*	No. of decoys	Cluster density
Model 1	0.76	0.82 ± 0.09	3.0 ± -2.1	9911	0.7306
Model 2	-4.11			134	0.0056
Model 3	-4.21			114	0.0051
Model 4	-3.24			255	0.0134
Model 5	-5.00			35	0.0017

* C-score is a confidence score for estimating the quality of predicted models by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5,2], where a C-score of higher value signifies a model with a high confidence and vice-versa.

TM-score and RMSD are known standards for measuring structural similarity between two structures which are usually used to measure the accuracy of structure modeling when the native structure is known. In case where the native structure is not known, it becomes necessary to predict the quality of the modeling prediction, i.e. what is the distance between the predicted model and the native structures? To answer this question, the TM-score and RMSD of the predicted models relative the native structures are determined based on the C-score.

Top 10 structural analogs in PDB

Figure E-2 – Alignment of predicted model (“model 1”) with structural analog 1d06A. Query structure is shown in cartoon, while the structural analog is displayed using backbone trace.

Table E-3 – Table listing top 10 structural analogs to predicted structure.

Rank	PDB Hit	TM-score	RMSD*	IDENa*	Cov*
1	1d06A	0.842	1.31	0.214	0.900
2	1y28A	0.840	0.89	0.265	0.869
3	1s67L	0.770	2.00	0.148	0.885
4	2vv7D	0.767	1.06	0.248	0.800
5	3lyxB	0.736	2.64	0.195	0.908
6	3k3cA	0.724	2.71	0.154	0.946
7	4hh2A	0.719	2.24	0.149	0.869
8	1v9yB	0.714	1.49	0.146	0.792
9	4gcZA1	0.713	3.05	0.198	0.931
10	4hiaA	0.708	2.93	0.130	0.908

*Ranking of proteins is based on TM-score of the structural alignment between the query structure and known structures in the PDB library. RMSDa is the RMSD between residues that are structurally aligned by TM-align. IDENa is the percentage sequence identity in the structurally aligned region. Cov represents the coverage of the alignment by TM-align and is equal to the number of structurally aligned residues divided by length of the query protein.

Predicted function using COACH

Ligand binding site

Figure E-3 – Predicted binding site residues and ligand of model 1. Grey cartoon represents cartoon of secondary structure of model 1, while blue sticks represent residues surrounding ligand. Green spheres represent the predicted ligand, heme. Details of the ligand binding site residues, Pdb hit and scores are listed in Table C-4.

Table E-4 – List of predicted ligands and associated ligand binding site residues of model 1

Rank	C-score*	Cluster size*	PDB Hit	Ligand name	Ligand Binding Site Residues
1	0.55	67	1dp6A	HEM (Heme b)	19, 21, 50, 53, 54, 61, 65, 68, 69, 71, 72, 77, 78, 79, 83, 85, 86, 97, 99, 101, 113, 114, 115
2	0.27	64	5hwwA	XBZ	17, 18, 19, 30, 50, 53, 54, 87, 99, 115, 116, 117
3	0.05	12	2V0WA	2V0WA00	19, 21, 50, 54, 61, 62, 63, 64, 65, 66, 67, 68, 69, 87, 113, 114, 115
4	0.03	9	3ve3A	HC4 (4-Coumaric Acid)	19, 30, 34, 38, 40, 50, 53, 55, 87, 88, 92
5	0.01	2	1s67L	OXY (dioxygen)	83, 99

* C-score is the confidence score of the prediction. C-score ranges [0-1], where a higher score indicates a more reliable prediction. Cluster size is the total number of templates in a cluster.

Sequence and structural alignment in pymol

Sequence alignment of model 1 and 1dp6

```

/1DP6//A/154      156 161 166 171 176 181 186 191      196 201
D-----AMIVIDGHGIIQLFSTAERLFGWSELEAIGQNIILMPE---PDRSFDSYI
/model1//A/1      6 11 16 21 26 31 36 41 46 51 56 61 66
DAERERAQITLASIGDGVITADTQGGISYLNPAEQMTNWTLDKARGLPASLFRIVDESSREEMLLI
206 211 216 221 226 231 236 241 246 251 256 261 266 500 719
SPYRTTSDDPHIIGIGRIVTGKRRDGTTFPHISIGEMQSGGE-PYATGVVDLTEHQQTQARLQEL OXY HEM
71 76 81 86 91 96 101 106 111 116 121 126
EQILSGEID--GGREHSLVLRHDGSSVPVTLVGAPIHRGAEITGVVLVLDMTREERQYMARL--- --- ---

```

Figure E-4 – Green residues reflect residues of 1dp6 while cyan residues reflect residues of model 1. Boxed residues reflect location of conserved histidine for heme binding.

Figure E-5 – Structural alignment of 1dp6 and model 1 in pymol displayed as secondary structure cartoon, Black lines represent heme group of 1dp6

Figure E-6 – Mesh representation of the surface of 1dp6 or the RbdA PAS predicted model 1. Two stick figures are superimposed in each image. The stick with green coloured backbone represent histidine from 1dp6 while the stick with a cyan coloured backbone represents glycine from model 1.

Sequence alignment of crystallized structures

Figure E-7 – Sequence alignment using crystal structure of RbdA and other proteins.

Appendix F

List of differentially expressed genes coding for PDEs and/or DGCs and proteins with PAS domains

PA number	Gene name	log2(fold-change)^							
		Bb	VS	Bb	VS	Ba	VS	Pb	VS
		Ba*		D*		D*		D*	
PA0169	<i>siaD</i>	-2.62							
PA0575	PA0575	1.15		1.09				1.89	
PA0861	<i>rbdA</i>	1.02				-0.87		0.54	
PA0873	<i>phhr</i>	1.70				-1.03			
PA1423	<i>bdlA</i>	1.99		1.38		-0.61		1.70	
PA1433	PA1433	-1.51		-1.18					0.83
PA1561	<i>aer</i>	1.27		0.72		-0.54		2.13	1.41
PA1930	PA1930	1.59		1.89				1.42	
PA2072	PA2072	1.04		1.62				0.71	
PA2177	PA2177	1.81		1.41				0.74	-0.67
PA2567	PA2567	0.75		0.80				2.04	1.24
PA2870	PA2870	1.26							
PA3177	PA3177	1.15				-1.57			
PA3271	PA3271	0.91							
PA3311	<i>nbdA</i>	1.62		1.63				1.90	
PA3702	<i>wspR</i>			1.02		1.17		1.29	
PA4021	PA4021	1.67				-0.92		1.60	
PA4112	PA4112					0.63		1.09	0.58
PA4293	<i>pprA</i>	1.83		2.21				3.24	
PA4601	<i>morA</i>							1.13	1.50
PA4929	PA4929	1.21		1.24					
PA5017	<i>dipA</i>	0.54		-0.51		-1.05			0.55
PA5484	<i>kinB</i>							-0.78	-1.10
PA5487	PA5487			-0.74					

* Where Bb refers to biofilm before NO treatment, D refers to planktonic dispersed cells, Ba refers to biofilm after NO treatment and Pb refers to planktonic cells before NO treatment.

[^] Log_2 fold change = Log_2 (FPKM value of sample listed first/ FPKM value of samples listed next). i.e. For Bb VS Ba, Log_2 fold change = Log_2 (FPKM Bb/ FPKM Ba).

References

Abdel-Mawgoud AM, Lépine F, Déziel E (2010). Rhamnolipids: diversity of structures, microbial origins and roles. *Applied Microbiology and Biotechnology* **86**: 1323-1336.

Alkawash MA, Soothill JS, Schiller NL (2006). Alginate lyase enhances antibiotic killing of mucoid *Pseudomonas aeruginosa* in biofilms. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* **114**: 131-138.

Alvarez L, Bricio C, Blesa A, Hidalgo A, Berenguer J (2014). Transferable Denitrification Capability of *Thermus thermophilus*. *Applied and Environmental Microbiology* **80**: 19-28.

Amikam D, Galperin MY (2006). PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics (Oxford, England)* **22**: 3-6.

An S, Wu J, Zhang LH (2010). Modulation of *Pseudomonas aeruginosa* biofilm dispersal by a cyclic-Di-GMP phosphodiesterase with a putative hypoxia-sensing domain. *Appl Environ Microbiol* **76**: 8160-8173.

Arai H, Hayashi M, Kuroi A, Ishii M, Igarashi Y (2005). Transcriptional Regulation of the Flavohemoglobin Gene for Aerobic Nitric Oxide Detoxification by the Second Nitric Oxide-Responsive Regulator of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **187**: 3960-3968.

Arai H (2011a). Regulation and Function of Versatile Aerobic and Anaerobic Respiratory Metabolism in *Pseudomonas aeruginosa*. *Frontiers in Microbiology* **2**.

Arai H (2011b). Regulation and Function of Versatile Aerobic and Anaerobic Respiratory Metabolism in *Pseudomonas aeruginosa*. *Frontiers in Microbiology* **2**: 103.

Arai H, Kawakami T, Osamura T, Hirai T, Sakai Y, Ishii M (2014). Enzymatic Characterization and *In vivo* Function of Five Terminal Oxidases in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **196**: 4206-4215.

Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME (2011). *Staphylococcus aureus* biofilms: Properties, regulation and roles in human disease. *Virulence* **2**: 445-459.

Arora DP, Hossain S, Xu Y, Boon EM (2015). Nitric Oxide Regulation of Bacterial Biofilms. *Biochemistry* **54**: 3717-3728.

Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP *et al* (2004). *Pseudomonas aeruginosa* Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and β -Lactamase and Alginate Production. *Antimicrobial Agents and Chemotherapy* **48**: 1175-1187.

Balfour-Lynn IM, Lavery A, Dinwiddie R (1996). Reduced upper airway nitric oxide in cystic fibrosis. *Archives of disease in childhood* **75**: 319-322.

Banin E, Vasil ML, Greenberg EP (2005). Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci USA* **102**: 11076-11081.

Banin E, Brady KM, Greenberg EP (2006). Chelator-Induced Dispersal and Killing of *Pseudomonas aeruginosa* Cells in a Biofilm. *Applied and Environmental Microbiology* **72**: 2064-2069.

Barnes RJ, Bandi RR, Wong WS, Barraud N, McDougald D, Fane A *et al* (2013). Optimal dosing regimen of nitric oxide donor compounds for the reduction of *Pseudomonas aeruginosa* biofilm and isolates from wastewater membranes. *Biofouling* **29**: 203-212.

Barnes RJ, Low JH, Bandi RR, Tay M, Chua F, Aung T *et al* (2015). Nitric Oxide Treatment for the Control of Reverse Osmosis Membrane Biofouling. *Applied and Environmental Microbiology* **81**: 2515-2524.

Barraud N, Hassett DJ, Hwang S-H, Rice SA, Kjelleberg S, Webb JS (2006). Involvement of Nitric Oxide in Biofilm Dispersal of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **188**: 7344-7353.

Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA *et al* (2009a). Nitric Oxide Signaling in *Pseudomonas aeruginosa* Biofilms Mediates Phosphodiesterase Activity, Decreased Cyclic Di-GMP Levels, and Enhanced Dispersal. *Journal of Bacteriology* **191**: 7333-7342.

- Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S (2009b). Nitric oxide - mediated dispersal in single - and multi - species biofilms of clinically and industrially relevant microorganisms. *Microbial biotechnology* **2**: 370-378.
- Barraud N, Kardak BG, Yepuri NR, Howlin RP, Webb JS, Faust SN *et al* (2012). Cephalosporin-3'-diazoniumdiolates: targeted NO-donor prodrugs for dispersing bacterial biofilms. *Angew Chem Int Ed Engl* **51**: 9057-9060.
- Barraud N, Moscoso JA, Ghigo J-M, Filloux A (2014). Methods for Studying Biofilm Dispersal in *Pseudomonas aeruginosa*. In: Filloux A, Ramos J-L (eds). *Pseudomonas Methods and Protocols*. Springer New York: New York, NY. pp 643-651.
- Barraud N, Kelso MJ, Rice SA, Kjelleberg S (2015). Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases. *Current pharmaceutical design* **21**: 31-42.
- Barrick D (1994). Replacement of the proximal ligand of sperm whale myoglobin with free imidazole in the mutant His-93-->Gly. *Biochemistry* **33**: 6546-6554.
- Basu Roy A, Sauer K (2014). Diguanylate cyclase NicD-based signalling mechanism of nutrient-induced dispersion by *Pseudomonas aeruginosa*. *Molecular microbiology* **94**: 771-793.
- Baty AM, Eastburn CC, Techkarnjanaruk S, Goodman AE, Geesey GG (2000). Spatial and Temporal Variations in Chitinolytic Gene Expression and Bacterial Biomass Production during Chitin Degradation. *Applied and Environmental Microbiology* **66**: 3574-3585.
- Bauer H, Jung T, Tsikas D, Stichtenoth DO, Frolich JC, Neumann C (1997). Nitric oxide inhibits the secretion of T-helper 1- and T-helper 2-associated cytokines in activated human T cells. *Immunology* **90**: 205-211.
- Bayer AS, Park S, Ramos MC, Nast CC, Eftekhari F, Schiller NL (1992). Effects of alginase on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa*. *Infection and Immunity* **60**: 3979-3985.

- Bertinaria M, Galli U, Sorba G, Fruttero R, Gasco A, Brenciaglia MI *et al* (2003). Synthesis and anti-*Helicobacter pylori* properties of NO-donor/metronidazole hybrids and related compounds. *Drug Dev Res* **60**: 225-239.
- Bethesda M (2007). Cystic Fibrosis Foundation Patient Registry 2006 Annual Data Report.
- Bhattacharjee A, Nusca TD, Hochbaum AI (2016). Rhamnolipids Mediate an Interspecies Biofilm Dispersal Signaling Pathway. *ACS Chemical Biology* **11**: 3068-3076.
- Bibikov SI, Biran R, Rudd KE, Parkinson JS (1997). A signal transducer for aerotaxis in *Escherichia coli*. *Journal of Bacteriology* **179**: 4075-4079.
- Blakytyn R, Jude E (2006). The molecular biology of chronic wounds and delayed healing in diabetes. *Diabetic Medicine* **23**: 594-608.
- Bodey GP, Bolivar R, Fainstein V, Jadeja L (1983). Infections caused by *Pseudomonas aeruginosa*. *Reviews of infectious diseases* **5**: 279-313.
- Bogdan C (2001a). Nitric oxide and the regulation of gene expression. *Trends in cell biology* **11**: 66-75.
- Bogdan C (2001b). Nitric oxide and the immune response. *Nature immunology* **2**: 907-916.
- Bogdan C (2015). Nitric oxide synthase in innate and adaptive immunity: an update. *Trends in immunology* **36**: 161-178.
- Bohnsack JF, Brown EJ (1986). The role of the spleen in resistance to infection. *Annual review of medicine* **37**: 49-59.
- Boles BR, Thoendel M, Singh PK (2005). Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Molecular microbiology* **57**: 1210-1223.
- Boles BR, Horswill AR (2008). agr-Mediated Dispersal of *Staphylococcus aureus* Biofilms. *PLoS Pathogens* **4**: e1000052.

- Boon EM, Davis JH, Tran R, Karow DS, Huang SH, Pan D *et al* (2006). Nitric Oxide Binding to Prokaryotic Homologs of the Soluble Guanylate Cyclase β 1 H-NOX Domain. *Journal of Biological Chemistry* **281**: 21892-21902.
- Boschi D, Tron GC, Lazzarato L, Chegaev K, Cena C, Di Stilo A *et al* (2006). NO-Donor Phenols: A New Class of Products Endowed with Antioxidant and Vasodilator Properties. *Journal of Medicinal Chemistry* **49**: 2886-2897.
- Bourlais CL, Acar L, Zia H, Sado PA, Needham T, Leverge R (1998). Ophthalmic drug delivery systems--recent advances. *Progress in retinal and eye research* **17**: 33-58.
- Bowler LL, Zhanel GG, Ball TB, Saward LL (2012). Mature *Pseudomonas aeruginosa* Biofilms Prevail Compared to Young Biofilms in the Presence of Ceftazidime. *Antimicrobial Agents and Chemotherapy* **56**: 4976-4979.
- Boyd A, Chakrabarty AM (1994). Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **60**: 2355-2359.
- Braxton EE, Jr., Ehrlich GD, Hall-Stoodley L, Stoodley P, Veeh R, Fux C *et al* (2005). Role of biofilms in neurosurgical device-related infections. *Neurosurgical review* **28**: 249-255.
- Bryers JD (2008). Medical Biofilms. *Biotechnology and bioengineering* **100**: 1-18.
- Butler CS, Seward HE, Greenwood C, Thomson AJ (1997). Fast Cytochrome bo from *Escherichia coli* Binds Two Molecules of Nitric Oxide at CuB. *Biochemistry* **36**: 16259-16266.
- Campbell JI, Ciofu O, Høiby N (1997). *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis have different beta-lactamase expression phenotypes but are homogeneous in the ampC-ampR genetic region. *Antimicrobial Agents and Chemotherapy* **41**: 1380-1384.
- Carlson HK, Vance RE, Marletta MA (2010). H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Molecular microbiology* **77**: 930-942.

- Cena C, Lolli ML, Lazzarato L, Guaita E, Morini G, Coruzzi G *et al* (2003). Antiinflammatory, Gastrosparing, and Antiplatelet Properties of New NO-Donor Esters of Aspirin. *Journal of Medicinal Chemistry* **46**: 747-754.
- Chalmers JM, Dent G (2006). Vibrational Spectroscopic Methods in Pharmaceutical Solid-state Characterization. *Polymorphism*. Wiley-VCH Verlag GmbH & Co. KGaA. pp 95-138.
- Chant A, Kraemer-Pecore CM, Watkin R, Kneale GG (2005). Attachment of a histidine tag to the minimal zinc finger protein of the *Aspergillus nidulans* gene regulatory protein AreA causes a conformational change at the DNA-binding site. *Protein expression and purification* **39**: 152-159.
- Charville GW, Hetrick EM, Geer CB, Schoenfisch MH (2008). Reduced bacterial adhesion to fibrinogen-coated substrates via nitric oxide release. *Biomaterials* **29**: 4039-4044.
- Chaurasia SS, Lim RR, Lakshminarayanan R, Mohan RR (2015). Nanomedicine Approaches for Corneal Diseases. *Journal of Functional Biomaterials* **6**: 277-298.
- Chegaev K, Riganti C, Lazzarato L, Rolando B, Guglielmo S, Campia I *et al* (2011). Nitric Oxide Donor Doxorubicins Accumulate into Doxorubicin-Resistant Human Colon Cancer Cells Inducing Cytotoxicity. *ACS Medicinal Chemistry Letters* **2**: 494-497.
- Chen F, Xia Q, Ju LK (2003). Aerobic denitrification of *Pseudomonas aeruginosa* monitored by online NAD(P)H fluorescence. *Appl Environ Microbiol* **69**: 6715-6722.
- Chen F, Xia Q, Ju LK (2006). Competition between oxygen and nitrate respirations in continuous culture of *Pseudomonas aeruginosa* performing aerobic denitrification. *Biotechnology and bioengineering* **93**: 1069-1078.
- Cherayil BJ, McCormick BA, Bosley J (2000). *Salmonella enterica* serovar typhimurium-dependent regulation of inducible nitric oxide synthase expression in macrophages by invasins SipB, SipC, and SipD and effector SopE2. *Infect Immun* **68**: 5567-5574.

Cherney DL, Amstutz HC (1983). Total hip replacement in the previously septic hip. *The Journal of bone and joint surgery American volume* **65**: 1256-1265.

Chmiel JF, Davis PB (2003). State of the art: why do the lungs of patients with cystic fibrosis become infected and why can't they clear the infection? *Respiratory research* **4**: 8.

Choi YC, Morgenroth E (2003). Monitoring biofilm detachment under dynamic changes in shear stress using laser-based particle size analysis and mass fractionation. *Water science and technology : a journal of the International Association on Water Pollution Research* **47**: 69-76.

Christen B, Christen M, Paul R, Schmid F, Folcher M, Jenoe P *et al* (2006). Allosteric control of cyclic di-GMP signaling. *J Biol Chem* **281**: 32015-32024.

Christen M, Christen B, Folcher M, Schauerte A, Jenal U (2005). Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* **280**: 30829-30837.

Christen M, Kulasekara HD, Christen B, Kulasekara BR, Hoffman LR, Miller SI (2010). Asymmetrical Distribution of the Second Messenger c-di-GMP upon Bacterial Cell Division. *Science (New York, NY)* **328**: 1295-1297.

Christensen LD, Moser C, Jensen PO, Rasmussen TB, Christophersen L, Kjelleberg S *et al* (2007). Impact of *Pseudomonas aeruginosa* quorum sensing on biofilm persistence in an *in vivo* intraperitoneal foreign-body infection model. *Microbiology (Reading, England)* **153**: 2312-2320.

Christensen LD, van Gennip M, Jakobsen TH, Alhede M, Hougen HP, Hoiby N *et al* (2012). Synergistic antibacterial efficacy of early combination treatment with tobramycin and quorum-sensing inhibitors against *Pseudomonas aeruginosa* in an intraperitoneal foreign-body infection mouse model. *The Journal of antimicrobial chemotherapy* **67**: 1198-1206.

Christensen LD, van Gennip M, Rybtke MT, Wu H, Chiang W-C, Alhede M *et al* (2013). Clearance of *Pseudomonas aeruginosa* Foreign-Body Biofilm Infections through Reduction of the Cyclic Di-GMP Level in the Bacteria. *Infection and Immunity* **81**: 2705-2713.

- Chua SL, Liu Y, Yam JKH, Chen Y, Vejborg RM, Tan BGC *et al* (2014). Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles **5**: 4462.
- Chua SL, Yam JKH, Hao P, Adav SS, Salido MM, Liu Y *et al* (2016). Selective labelling and eradication of antibiotic-tolerant bacterial populations in *Pseudomonas aeruginosa* biofilms. *Nature Communications* **7**: 10750.
- Ciofu O (2003). *Pseudomonas aeruginosa* chromosomal beta-lactamase in patients with cystic fibrosis and chronic lung infection. Mechanism of antibiotic resistance and target of the humoral immune response. *APMIS Supplementum*: 1-47.
- Ciofu O, Riis B, Pressler T, Poulsen HE, Høiby N (2005). Occurrence of Hypermutable *Pseudomonas aeruginosa* in Cystic Fibrosis Patients Is Associated with the Oxidative Stress Caused by Chronic Lung Inflammation. *Antimicrobial Agents and Chemotherapy* **49**: 2276-2282.
- Coleman JW (2001). Nitric oxide in immunity and inflammation. *International immunopharmacology* **1**: 1397-1406.
- Collins SA, Kelso MJ, Rineh A, Yepuri NR, Coles J, Jackson CL *et al* (2017). Cephalosporin-3'-Diazeniumdiolate NO Donor Prodrug PYRRO-C3D Enhances Azithromycin Susceptibility of Nontypeable *Haemophilus influenzae* Biofilms. *Antimicrobial Agents and Chemotherapy* **61**.
- Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GCL *et al* (2011). The Pel Polysaccharide Can Serve a Structural and Protective Role in the Biofilm Matrix of *Pseudomonas aeruginosa*. *PLOS Pathogens* **7**: e1001264.
- Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C *et al* (2012). The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environmental microbiology* **14**: 10.1111/j.1462-2920.2011.02657.x.
- Comolli JC, Donohue TJ (2004). Differences in two *Pseudomonas aeruginosa* *cbb3* cytochrome oxidases. *Molecular microbiology* **51**: 1193-1203.

- Cowley ES, Kopf SH, LaRiviere A, Ziebis W, Newman DK (2015). Pediatric Cystic Fibrosis Sputum Can Be Chemically Dynamic, Anoxic, and Extremely Reduced Due to Hydrogen Sulfide Formation. *mBio* **6**: e00767-00715.
- Cox CD, Adams P (1985). Siderophore activity of pyoverdinin for *Pseudomonas aeruginosa*. *Infect Immun* **48**: 130-138.
- Cozzzone AJ (1981). How do bacteria synthesize proteins during amino acid starvation? *Trends in Biochemical Sciences* **6**: 108-110.
- Cunningham L, Williams HD (1995). Isolation and characterization of mutants defective in the cyanide-insensitive respiratory pathway of *Pseudomonas aeruginosa*. *J Bacteriol* **177**: 432-438.
- Cunningham L, Pitt M, Williams HD (1997). The *cioAB* genes from *Pseudomonas aeruginosa* code for a novel cyanide-insensitive terminal oxidase related to the cytochrome bd quinol oxidases. *Molecular microbiology* **24**: 579-591.
- Cutruzzola F, Frankenberg-Dinkel N (2015). Origin and impact of nitric oxide in *Pseudomonas aeruginosa* biofilms. *Journal of Bacteriology*.
- D'Autreaux B, Touati D, Bersch B, Latour JM, Michaud-Soret I (2002). Direct inhibition by nitric oxide of the transcriptional ferric uptake regulation protein via nitrosylation of the iron. *Proc Natl Acad Sci U S A* **99**: 16619-16624.
- Davenport EK, Call DR, Beyenal H (2014). Differential Protection from Tobramycin by Extracellular Polymeric Substances from *Acinetobacter baumannii* and *Staphylococcus aureus* Biofilms. *Antimicrobial Agents and Chemotherapy* **58**: 4755-4761.
- Davey ME, Caiazza NC, O'Toole GA (2003). Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1. *Journal of Bacteriology* **185**: 1027-1036.
- Davies DG, Marques CNH (2009). A Fatty Acid Messenger Is Responsible for Inducing Dispersion in Microbial Biofilms. *Journal of Bacteriology* **191**: 1393-1403.

- de Beer D, Stoodley P, Lewandowski Z (1997). Measurement of local diffusion coefficients in biofilms by microinjection and confocal microscopy. *Biotechnology and bioengineering* **53**: 151-158.
- De Groote MA, Fang FC (1995). NO inhibitions: antimicrobial properties of nitric oxide. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **21 Suppl 2**: S162-165.
- Dibdin GH, Assinder SJ, Nichols WW, Lambert PA (1996). Mathematical model of beta-lactam penetration into a biofilm of *Pseudomonas aeruginosa* while undergoing simultaneous inactivation by released beta-lactamases. *The Journal of antimicrobial chemotherapy* **38**: 757-769.
- Dong Y-H, Zhang X-F, An S-W, Xu J-L, Zhang L-H (2008). A novel two-component system BqsS-BqsR modulates quorum sensing-dependent biofilm decay in *Pseudomonas aeruginosa*. *Communicative & Integrative Biology* **1**: 88-96.
- Dow JM, Crossman L, Findlay K, He Y-Q, Feng J-X, Tang J-L (2003). Biofilm dispersal in *Xanthomonas campestris* is controlled by cell–cell signaling and is required for full virulence to plants. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 10995-11000.
- Driffield K, Miller K, Bostock JM, O'Neill AJ, Chopra I (2008). Increased mutability of *Pseudomonas aeruginosa* in biofilms. *Journal of Antimicrobial Chemotherapy* **61**: 1053-1056.
- Duong HTT, Adnan NNM, Barraud N, Basuki JS, Kutty SK, Jung K *et al* (2014). Functional gold nanoparticles for the storage and controlled release of nitric oxide: applications in biofilm dispersal and intracellular delivery. *Journal of Materials Chemistry B* **2**: 5003-5011.
- Estahbanati HK, Kashani PP, Ghanaatpisheh F (2002). Frequency of *Pseudomonas aeruginosa* serotypes in burn wound infections and their resistance to antibiotics. *Burns : journal of the International Society for Burn Injuries* **28**: 340-348.
- Fang Y-I, Ohata H, Honda K (2009). Fluorometric determination of nitrite with 2,3-diaminonaphthalene by reverse phase HPLC under alkaline conditions. *J Pharmacol Toxicol Methods* **59**: 153-155.

- Feelisch M, Schonafinger K, Noack E (1992a). Thiol-mediated generation of nitric oxide accounts for the vasodilator action of furoxans. *Biochemical Pharmacology* **44**: 1149-1157.
- Feelisch M, Schönafingeri K, Noack H (1992b). Thiol-mediated generation of nitric oxide accounts for the vasodilator action of furoxans. *Biochemical Pharmacology* **44**: 1149-1157.
- Ferrero R, Rodríguez-Pascual F, Miras-Portugal MT, Torres M (1999). Comparative effects of several nitric oxide donors on intracellular cyclic GMP levels in bovine chromaffin cells: correlation with nitric oxide production. *British Journal of Pharmacology* **127**: 779-787.
- Filiatrault MJ, Picardo KF, Ngai H, Passador L, Iglewski BH (2006). Identification of *Pseudomonas aeruginosa* Genes Involved in Virulence and Anaerobic Growth. *Infection and Immunity* **74**: 4237-4245.
- Finkelstein RA, Boesman-Finkelstein M, Chang Y, Häse CC (1992). *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. *Infection and Immunity* **60**: 472-478.
- Ford PC, Wink DA, Stanbury DM (1993). Autoxidation kinetics of aqueous nitric oxide. *FEBS Letters* **326**: 1-3.
- Frei R, Breitbach AS, Blackwell HE (2012). 2-Aminobenzimidazole Derivatives Strongly Inhibit and Disperse *Pseudomonas aeruginosa* Biofilms. *Angewandte Chemie (International ed in English)* **51**: 5226-5229.
- Friedrichsen W (2001). *Monocyclic furazans and furoxans*, vol. 78.
- Fruttero R, Crosetti M, Chegaev K, Guglielmo S, Gasco A, Berardi F *et al* (2010). Phenylsulfonylfuroxans as Modulators of Multidrug-Resistance-Associated Protein-1 and P-Glycoprotein. *Journal of Medicinal Chemistry* **53**: 5467-5475.
- Fuda C, Suvorov M, Vakulenko SB, Mobashery S (2004). The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *J Biol Chem* **279**: 40802-40806.

- Gales AC, Jones RN, Turnidge J, Rennie R, Ramphal R (2001). Characterization of *Pseudomonas aeruginosa* isolates: occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the global SENTRY Antimicrobial Surveillance Program, 1997-1999. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **32 Suppl 2**: S146-155.
- Galperin MY, Gaidenko TA, Mulkidjanian AY, Nakano M, Price CW (2001). MHYT, a new integral membrane sensor domain. *FEMS Microbiol Lett* **205**: 17-23.
- Gambello MJ, Iglewski BH (1991). Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *Journal of Bacteriology* **173**: 3000-3009.
- Gao JJ, Zuvanich EG, Xue Q, Horn DL, Silverstein R, Morrison DC (1999). Cutting Edge: Bacterial DNA and LPS Act in Synergy in Inducing Nitric Oxide Production in RAW 264.7 Macrophages. *The Journal of Immunology* **163**: 4095.
- Garcia D, Orillard E, Johnson MS, Watts KJ (2017). Gas sensing and signalling in the PAS-heme domain of the *Pseudomonas aeruginosa* Aer2 receptor. *Journal of Bacteriology*.
- Gardner PR, Gardner AM, Martin LA, Salzman AL (1998). Nitric oxide dioxygenase: An enzymic function for flavohemoglobin. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 10378-10383.
- Gasco A, Fruttero R, Sorba G, Di Stilo A, R C (2004). NO donors: Focus on furoxan derivatives. *Pure Applied Chemistry* **76**: 973 - 981.
- Gasco A, Schoenafinger K (2005). The NO-Releasing Heterocycles. *Nitric Oxide Donors*. Wiley-VCH Verlag GmbH & Co. KGaA. pp 131-175.
- Gaynes R, Edwards JR (2005). Overview of nosocomial infections caused by gram-negative bacilli. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **41**: 848-854.
- Ghaffari A, Jalili R, Ghaffari M, Miller C, Ghahary A (2007). Efficacy of gaseous nitric oxide in the treatment of skin and soft tissue infections. *Wound repair and*

regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society **15**: 368-377.

Ghigo D, Calvino R, Heller R, Calvino R, Alessio P, Fruttero R *et al* (1992). Characterization of a new compound, S35b, as a guanylate cyclase activator in human platelets. *Biochemical Pharmacology* **43**: 1281-1288.

Giardina G, Rinaldo S, Johnson KA, Di Matteo A, Brunori M, Cutruzzola F (2008). NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *Journal of molecular biology* **378**: 1002-1015.

Gilles-Gonzalez MA, Gonzalez G, Perutz MF, Kiger L, Marden MC, Poyart C (1994). Heme-Based Sensors, Exemplified by the Kinase FixL, Are a New Class of Heme Protein with Distinctive Ligand Binding and Autoxidation. *Biochemistry* **33**: 8067-8073.

Gilles-Gonzalez MA, Gonzalez G (2004). Signal transduction by heme-containing PAS-domain proteins. *Journal of applied physiology (Bethesda, Md : 1985)* **96**: 774-783.

Givskov M, Eberl L, Moller S, Poulsen LK, Molin S (1994). Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. *J Bacteriol* **176**: 7-14.

Giwerzman B, Jensen ET, Hoiby N, Kharazmi A, Costerton JW (1991). Induction of beta-lactamase production in *Pseudomonas aeruginosa* biofilm. *Antimicrob Agents Chemother* **35**: 1008-1010.

Gjermansen M, Ragas P, Sternberg C, Molin S, Tolker-Nielsen T (2005). Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environ Microbiol* **7**: 894-904.

Gjermansen M, Nilsson M, Yang L, Tolker-Nielsen T (2010). Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: genetic elements and molecular mechanisms. *Molecular microbiology* **75**: 815-826.

Gokhale NS (2008). Medical management approach to infectious keratitis. *Indian Journal of Ophthalmology* **56**: 215-220.

Gong W, Hao B, Mansy SS, Gonzalez G, Gilles-Gonzalez MA, Chan MK (1998). Structure of a biological oxygen sensor: A new mechanism for heme-driven signal transduction. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 15177-15182.

Grasemann H, Michler E, Wallot M, Ratjen F (1997). Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. *Pediatric pulmonology* **24**: 173-177.

Grasemann H, Grasemann C, Kurtz F, Tietze-Schillings G, Vester U, Ratjen F (2005). Oral L-arginine supplementation in cystic fibrosis patients: a placebo-controlled study. *The European respiratory journal* **25**: 62-68.

Grisham MB, Johnson GG, Lancaster JR (1996). Quantitation of nitrate and nitrite in extracellular fluids. *Methods in Enzymology*. Academic Press. pp 237-246.

Groat RG, Schultz JE, Zychlinsky E, Bockman A, Matin A (1986). Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. *Journal of Bacteriology* **168**: 486-493.

Guerinot ML (1994). Microbial Iron Transport. *Annu Rev Microbiol* **48**: 743-772.

Hall-Stoodley L, Costerton JW, Stoodley P (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature reviews Microbiology* **2**: 95-108.

Hall-Stoodley L, Stoodley P (2005). Biofilm formation and dispersal and the transmission of human pathogens. *Trends in microbiology* **13**: 7-10.

Hamad B (2010). The antibiotics market. *Nature reviews Drug discovery* **9**: 675-676.

Hammond JH, Dolben EF, Smith TJ, Bhuju S, Hogan DA (2015). Links between Anr and Quorum Sensing in *Pseudomonas aeruginosa* Biofilms. *Journal of Bacteriology* **197**: 2810-2820.

Hampl V, Herget J (2000). Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. *Physiological reviews* **80**: 1337-1372.

- Härtig E, Zumft WG (1998). The Requirement of RpoN (Sigma Factor $\zeta(54)$) in Denitrification by *Pseudomonas stutzeri* Is Indirect and Restricted to the Reduction of Nitrite and Nitric Oxide. *Applied and Environmental Microbiology* **64**: 3092-3095.
- Hattori Y, Nakanishi N, Kasai K, Shimoda S-I (1997). GTP cyclohydrolase I mRNA induction and tetrahydrobiopterin synthesis in human endothelial cells. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1358**: 61-66.
- He Y, Maisuradze GG, Yin Y, Kachlishvili K, Rackovsky S, Scheraga HA (2017). Sequence-, structure-, and dynamics-based comparisons of structurally homologous CheY-like proteins. *Proceedings of the National Academy of Sciences* **114**: 1578-1583.
- Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR *et al* (2002). Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology (Reading, England)* **148**: 87-102.
- Hernandez-Cuellar E, Tsuchiya K, Hara H, Fang R, Sakai S, Kawamura I *et al* (2012). Cutting Edge: Nitric Oxide Inhibits the NLRP3 Inflammasome. *The Journal of Immunology* **189**: 5113.
- Hetrick EM, Shin JH, Stasko NA, Johnson CB, Wespe DA, Holmuhamedov E *et al* (2008). Bactericidal efficacy of nitric oxide-releasing silica nanoparticles. *ACS nano* **2**: 235-246.
- Hickman JW, Harwood CS (2008). Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Molecular microbiology* **69**: 376-389.
- Hider RC, Kong X (2010). Chemistry and biology of siderophores. *Natural Product Reports* **27**: 637-657.
- Hill S, Austin S, Eydmann T, Jones T, Dixon R (1996). *Azotobacter vinelandii* NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 2143-2148.

- Hironaka I, Iwase T, Sugimoto S, Okuda K-i, Tajima A, Yanaga K *et al* (2013). Glucose Triggers ATP Secretion from Bacteria in a Growth-Phase-Dependent Manner. *Appl Environ Microbiol* **79**: 2328-2335.
- Hofseth LJ, Saito Si, Hussain SP, Espey MG, Miranda KM, Araki Y *et al* (2003). Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 143-148.
- Højby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010). Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents* **35**: 322-332.
- Hori H, Tsubaki M, Mogi T, Anraku Y (1996). EPR study of NO complex of bd-type ubiquinol oxidase from *Escherichia coli*. *J Biol Chem* **271**: 9254-9258.
- Hossain S, Boon EM (2017). Discovery of a Novel Nitric Oxide Binding Protein and Nitric-Oxide-Responsive Signaling Pathway in *Pseudomonas aeruginosa*. *ACS infectious diseases* **3**: 454-461.
- Hottinger DG, Beebe DS, Kozhimannil T, Prielipp RC, Belani KG (2014). Sodium nitroprusside in 2014: A clinical concepts review. *Journal of Anaesthesiology, Clinical Pharmacology* **30**: 462-471.
- Howlin R, Cathie K, Hall-Stoodley L, Niehaus L, Connett G, Legg J *et al* (2011). Nitric oxide-mediated dispersal and enhanced antibiotic sensitivity in *Pseudomonas aeruginosa* biofilms from the cystic fibrosis lung. *Archives of disease in childhood* **96**: A45.
- Howlin RP, Cathie K, Hall-Stoodley L, Cornelius V, Duignan C, Allan RN *et al* (2017). Low-Dose Nitric Oxide as Targeted Anti-biofilm Adjunctive Therapy to Treat Chronic *Pseudomonas aeruginosa* Infection in Cystic Fibrosis. *Molecular Therapy*.
- Hrabie JA, Klose JR, Wink DA, Keefer LK (1993). New nitric oxide-releasing zwitterions derived from polyamines. *The Journal of Organic Chemistry* **58**: 1472-1476.

- Hugo C, Williams P (2005). Pharmacological Properties of Furoxans and Benzofuroxans: Recent Developments. *Mini-Reviews in Medicinal Chemistry* **5**: 57-71.
- Huynh TT, McDougald D, Klebensberger J, Al Qarni B, Barraud N, Rice SA *et al* (2012). Glucose Starvation-Induced Dispersal of *Pseudomonas aeruginosa* Biofilms Is cAMP and Energy Dependent. *PLOS ONE* **7**: e42874.
- Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE (1993). Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 8103-8107.
- Irie Y, O'Toole G A, Yuk MH (2005). *Pseudomonas aeruginosa* rhamnolipids disperse *Bordetella bronchiseptica* biofilms. *FEMS Microbiol Lett* **250**: 237-243.
- Jackson DW, Suzuki K, Oakford L, Simecka JW, Hart ME, Romeo T (2002). Biofilm Formation and Dispersal under the Influence of the Global Regulator CsrA of *Escherichia coli*. *Journal of Bacteriology* **184**: 290-301.
- Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S *et al* (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences* **100**: 14339-14344.
- Jacoby GA, Munoz-Price LS (2005). The new beta-lactamases. *The New England journal of medicine* **352**: 380-391.
- Jacoby GA (2009). AmpC beta-lactamases. *Clin Microbiol Rev* **22**: 161-182, Table of Contents.
- Jang I-A, Kim J, Park W (2016). Endogenous hydrogen peroxide increases biofilm formation by inducing exopolysaccharide production in *Acinetobacter oleivorans* DR1. *Scientific Reports* **6**: 21121.
- Jardeleza C, Foreman A, Baker L, Paramasivan S, Field J, Tan LW *et al* (2011). The effects of nitric oxide on *Staphylococcus aureus* biofilm growth and its implications in chronic rhinosinusitis. *International forum of allergy & rhinology* **1**: 438-444.

- Jean D, Maitre B, Tankovic J, Meignan M, Adnot S, Brun-Buisson C *et al* (2002). Beneficial effects of nitric oxide inhalation on pulmonary bacterial clearance. *Critical care medicine* **30**: 442-447.
- Jefferson KK, Goldmann DA, Pier GB (2005). Use of Confocal Microscopy To Analyze the Rate of Vancomycin Penetration through *Staphylococcus aureus* Biofilms. *Antimicrobial Agents and Chemotherapy* **49**: 2467-2473.
- Jenal U, Malone J (2006). Mechanisms of cyclic-di-GMP signaling in bacteria. *Annual review of genetics* **40**: 385-407.
- Jensen V, Löns D, Zaoui C, Bredenbruch F, Meissner A, Dieterich G *et al* (2006). RhlR Expression in *Pseudomonas aeruginosa* Is Modulated by the Pseudomonas Quinolone Signal via PhoB-Dependent and -Independent Pathways. *Journal of Bacteriology* **188**: 8601-8606.
- Kaplan JB, Meyenhofer MF, Fine DH (2003). Biofilm Growth and Detachment of *Actinobacillus actinomycetemcomitans*. *Journal of Bacteriology* **185**: 1399-1404.
- Kaplan JB (2010). Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. *Journal of Dental Research* **89**: 205-218.
- Katrina C, Robert H, Nicolas B, Mary C, Stuart C, Gary C *et al* (2014). Low Dose Nitric Oxide As Adjunctive Therapy To Reduce Antimicrobial Tolerance Of Pseudomonas Aeruginosa Biofilms In The Treatment Of Patients With Cystic Fibrosis: Report Of A Proof Of Concept Clinical Trial. B38. *UPDATE IN ADULT CYSTIC FIBROSIS*. American Thoracic Society. pp A2843-A2843.
- Katritzky AR, Boulton AJ (1982). *Advances in Heterocyclic Chemistry*. Elsevier Science.
- Kawakami T, Kuroki M, Ishii M, Igarashi Y, Arai H (2010). Differential expression of multiple terminal oxidases for aerobic respiration in *Pseudomonas aeruginosa*. *Environ Microbiol* **12**: 1399-1412.
- Kazmierczak BI, Lebron MB, Murray TS (2006). Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. *Molecular microbiology* **60**: 1026-1043.

- Keefer LK (2011). Fifty Years of Diazeniumdiolate Research. From Laboratory Curiosity to Broad-Spectrum Biomedical Advances. *ACS Chemical Biology* **6**: 1147-1155.
- Ketola T, Mikonranta L, Laakso J, Mappes J (2016). Different food sources elicit fast changes to bacterial virulence. *Biology Letters* **12**.
- Kim BJ, Park JH, Park TH, Bronstein PA, Schneider DJ, Cartinhour SW *et al* (2009). Effect of Iron Concentration on the Growth Rate of *Pseudomonas syringae* and the Expression of Virulence Factors in hrp-Inducing Minimal Medium. *Appl Environ Microbiol* **75**: 2720-2726.
- Klockgether J, Munder A, Neugebauer J, Davenport CF, Stanke F, Larbig KD *et al* (2010). Genome Diversity of *Pseudomonas aeruginosa* PAO1 Laboratory Strains. *Journal of Bacteriology* **192**: 1113-1121.
- Knowles R (1982). Denitrification. *Microbiological Reviews* **46**: 43-70.
- Koh KS, Lam KW, Alhede M, Queck SY, Labbate M, Kjelleberg S *et al* (2007). Phenotypic Diversification and Adaptation of *Serratia marcescens* MG1 Biofilm-Derived Morphotypes. *Journal of Bacteriology* **189**: 119-130.
- Kohler T, Michea-Hamzehpour M, Epp SF, Pechere JC (1999). Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. *Antimicrob Agents Chemother* **43**: 424-427.
- Kollef MH, Shorr A, Tabak YP, Gupta V, Liu LZ, Johannes RS (2005). Epidemiology and outcomes of health-care-associated pneumonia: results from a large US database of culture-positive pneumonia. *Chest* **128**: 3854-3862.
- Korfmann G, Wiedemann B (1988). Genetic control of beta-lactamase production in *Enterobacter cloacae*. *Reviews of infectious diseases* **10**: 793-799.
- Koskenkorva-Frank TS, Weiss G, Koppenol WH, Burckhardt S (2013). The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: insights into the potential of various iron therapies to induce oxidative and nitrosative stress. *Free radical biology & medicine* **65**: 1174-1194.
- Kubes P, Suzuki M, Granger DN (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* **88**: 4651-4655.

Kulasekara BR, Kamischke C, Kulasekara HD, Christen M, Wiggins PA, Miller SI (2013). c-di-GMP heterogeneity is generated by the chemotaxis machinery to regulate flagellar motility. *eLife* **2**: e01402.

Kulasekara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S *et al* (2006). Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 2839-2844.

Kutty SK, Barraud N, Pham A, Iskander G, Rice SA, Black DS *et al* (2013). Design, Synthesis, and Evaluation of Fimbrilide–Nitric Oxide Donor Hybrids as Antimicrobial Agents. *Journal of Medicinal Chemistry* **56**: 9517-9529.

Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D (2004). *Pseudomonas aeruginosa* Pyocyanin Is Critical for Lung Infection in Mice. *Infection and Immunity* **72**: 4275-4278.

Lawrence JR, Scharf B, Packroff G, Neu TR (2002). Microscale evaluation of the effects of grazing by invertebrates with contrasting feeding modes on river biofilm architecture and composition. *Microbial ecology* **44**: 199-207.

Lechevallier M (2003). *Conditions favouring coliform and HPC bacterial growth in drinking water and on water contact surfaces*.

Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J *et al* (2013). A cell-cell communication signal integrates quorum sensing and stress response. *Nature chemical biology* **9**: 339-343.

Lee VT, Matewish JM, Kessler JL, Hyodo M, Hayakawa Y, Lory S (2007). A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Molecular microbiology* **65**: 1474-1484.

Lequette Y, Greenberg EP (2005). Timing and Localization of Rhamnolipid Synthesis Gene Expression in *Pseudomonas aeruginosa* Biofilms. *Journal of Bacteriology* **187**: 37-44.

- Lequette Y, Boels G, Clarisse M, Faille C (2010). Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. *Biofouling* **26**: 421-431.
- Lewis K (2007). Persister cells, dormancy and infectious disease. *Nat Rev Micro* **5**: 48-56.
- Li Y-H, Tian X (2012). Quorum Sensing and Bacterial Social Interactions in Biofilms. *Sensors (Basel, Switzerland)* **12**: 2519-2538.
- Li Y, Heine S, Entian M, Sauer K, Frankenberg-Dinkel N (2013a). NO-Induced Biofilm Dispersion in *Pseudomonas aeruginosa* Is Mediated by an MHYT Domain-Coupled Phosphodiesterase. *Journal of Bacteriology* **195**: 3531-3542.
- Li Y, Heine S, Entian M, Sauer K, Frankenberg-Dinkel N (2013b). NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J Bacteriol* **195**: 3531-3542.
- Li Y, Petrova OE, Su S, Lau GW, Panmanee W, Na R *et al* (2014). BdlA, DipA and induced dispersion contribute to acute virulence and chronic persistence of *Pseudomonas aeruginosa*. *PLoS Pathog* **10**: e1004168.
- Li Z, Chen J-H, Hao Y, Nair SK (2012). Structures of the PelD Cyclic Diguanylate Effector Involved in Pellicle Formation in *Pseudomonas aeruginosa* PAO1. *The Journal of Biological Chemistry* **287**: 30191-30204.
- Lin M-H, Shu J-C, Huang H-Y, Cheng Y-C (2012). Involvement of Iron in Biofilm Formation by *Staphylococcus aureus*. *PLOS ONE* **7**: e34388.
- Lindell MJ, Granéli W, Tranvik LJ (1995). Enhanced bacterial growth in response to photochemical transformation of dissolved organic matter. *Limnol Oceanogr* **40**: 195-199.
- Lister PD, Wolter DJ, Hanson ND (2009a). Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* **22**: 582-610.
- Lister PD, Wolter DJ, Hanson ND (2009b). Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of

Chromosomally Encoded Resistance Mechanisms. *Clinical Microbiology Reviews* **22**: 582-610.

Liu C, Liew CW, Wong YH, Tan ST, Poh WH, Manimekalai SMS *et al* (2017). Insights into biofilm dispersal regulation from the crystal structure of the PAS-GGDEF-EAL region of RbdA from *Pseudomonas aeruginosa*. *Journal of Bacteriology*.

Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA *et al* (2012). Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry* **51**: 2087-2099.

Livermore DM (2009). beta-Lactamases- the Threat Renews. *Current protein & peptide science* **10**: 397-400.

Llanes C, Pourcel C, Richardot C, Plesiat P, Fichant G, Cavallo JD *et al* (2013). Diversity of beta-lactam resistance mechanisms in cystic fibrosis isolates of *Pseudomonas aeruginosa*: a French multicentre study. *The Journal of antimicrobial chemotherapy* **68**: 1763-1771.

Lloyd-Jones DM, Bloch KD (1996). The vascular biology of nitric oxide and its role in atherogenesis. *Annual review of medicine* **47**: 365-375.

Loftsson T (2014). Chapter 3 - Degradation Pathways. *Drug Stability for Pharmaceutical Scientists*. Academic Press: San Diego. pp 63-104.

Lopez-Sanchez A, Jimenez-Fernandez A, Calero P, Gallego LD, Govantes F (2013). New methods for the isolation and characterization of biofilm-persistent mutants in *Pseudomonas putida*. *Environmental microbiology reports* **5**: 679-685.

Lopez N, Kobayashi L, Coimbra R (2011). A Comprehensive review of abdominal infections. *World Journal of Emergency Surgery : WJES* **6**: 7-7.

Luyt C-E, Aubry A, Lu Q, Micaelo M, Bréchet N, Brossier F *et al* (2014). Imipenem, Meropenem, or Doripenem To Treat Patients with *Pseudomonas aeruginosa* Ventilator-Associated Pneumonia. *Antimicrobial Agents and Chemotherapy* **58**: 1372-1380.

- Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ (2009). Assembly and Development of the *Pseudomonas aeruginosa* Biofilm Matrix. *PLoS Pathogens* **5**: e1000354.
- Ma Q, Yang Z, Pu M, Peti W, Wood TK (2011). Engineering a Novel c-di-GMP-Binding Protein for Biofilm Dispersal. *Environmental microbiology* **13**: 631-642.
- Macheroux P, Hill S, Austin S, Eydmann T, Jones T, Kim SO *et al* (1998). Electron donation to the flavoprotein NifL, a redox-sensing transcriptional regulator. *Biochemical Journal* **332**: 413-419.
- Mai-Prochnow A, Lucas-Elio P, Egan S, Thomas T, Webb JS, Sanchez-Amat A *et al* (2008). Hydrogen Peroxide Linked to Lysine Oxidase Activity Facilitates Biofilm Differentiation and Dispersal in Several Gram-Negative Bacteria. *Journal of Bacteriology* **190**: 5493-5501.
- Mancl KA, Kirsner RS, Ajdic D (2013). Wound biofilms: lessons learned from oral biofilms. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **21**: 352-362.
- Manefield M, Rasmussen TB, Henzter M, Andersen JB, Steinberg P, Kjelleberg S *et al* (2002). Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology (Reading, England)* **148**: 1119-1127.
- Mao K, Chen S, Chen M, Ma Y, Wang Y, Huang B *et al* (2013). Nitric oxide suppresses NLRP3 inflammasome activation and protects against LPS-induced septic shock. *Cell Res* **23**: 201-212.
- Marin J, Rodriguez-Martinez MA (1997). Role of vascular nitric oxide in physiological and pathological conditions. *Pharmacology & therapeutics* **75**: 111-134.
- Marshall HE, Merchant K, Stamler JS (2000). Nitrosation and oxidation in the regulation of gene expression. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **14**: 1889-1900.
- Marvasi M, Chen C, Carrazana M, Durie IA, Teplitski M (2014). Systematic analysis of the ability of Nitric Oxide donors to dislodge biofilms formed by *Salmonella enterica* and *Escherichia coli* O157:H7. *AMB Express* **4**: 42-42.

Marvasi M, Durie IA, Henríquez T, Satkute A, Matuszewska M, Prado RC (2016). Dispersal of human and plant pathogens biofilms via nitric oxide donors at 4 °C. *AMB Express* **6**: 49.

Matthews JR, Botting CH, Panico M, Morris HR, Hay RT (1996). Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic acids research* **24**: 2236-2242.

McCollister BD, Hoffman M, Husain M, Vázquez-Torres A (2011). Nitric Oxide Protects Bacteria from Aminoglycosides by Blocking the Energy-Dependent Phases of Drug Uptake. *Antimicrobial Agents and Chemotherapy* **55**: 2189-2196.

Medana C, Ermondi G, Fruttero R, Di Stilo A, Ferretti C, Gasco A (1994). Furoxans as Nitric Oxide Donors. 4-Phenyl-3-furoxancarbonitrile: Thiol-Mediated Nitric Oxide Release and Biological Evaluation. *Journal of Medicinal Chemistry* **37**: 4412-4416.

Mena KD, Gerba CP (2009). Risk assessment of *Pseudomonas aeruginosa* in water. *Reviews of environmental contamination and toxicology* **201**: 71-115.

Meng QH, Springall DR, Bishop AE, Morgan K, Evans TJ, Habib S *et al* (1998). Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. *The Journal of pathology* **184**: 323-331.

Merighi M, Lee VT, Hyodo M, Hayakawa Y, Lory S (2007). The second messenger bis-(3'-5')-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Molecular microbiology* **65**: 876-895.

Messenger AJM, Barclay R (1983). Bacteria, iron and pathogenicity. *Biochemical Education* **11**: 54-63.

Michl RK, Hentschel J, Fischer C, Beck JF, Mainz JG (2013). Reduced Nasal Nitric Oxide Production in Cystic Fibrosis Patients with Elevated Systemic Inflammation Markers. *PLOS ONE* **8**: e79141.

Miles AM, Wink DA, Cook JC, Grisham MB (1996a). Determination of nitric oxide using fluorescence spectroscopy. *Methods in Enzymology*. Academic Press. pp 105-120.

Miles AM, Wink DA, Cook JC, Grisham MB (1996b). Determination of nitric oxide using fluorescence spectroscopy. *Methods in enzymology* **268**: 105-120.

Miller CC, Miller MK, Ghaffari A, Kunimoto B (2004). Treatment of chronic nonhealing leg ulceration with gaseous nitric oxide: a case study. *Journal of cutaneous medicine and surgery* **8**: 233-238.

Miller CC, Hergott CA, Rohan M, Arsenault-Mehta K, Döring G, Mehta S (2013). Inhaled nitric oxide decreases the bacterial load in a rat model of *Pseudomonas aeruginosa* pneumonia. *Journal of Cystic Fibrosis* **12**: 817-820.

Miller MR, Megson IL (2007). Recent developments in nitric oxide donor drugs. *British Journal of Pharmacology* **151**: 305-321.

Mimura H, Gato K, Kitamura S, Kitagawa T, Kohda S (2002). Effect of water content on the solid-state stability in two isomorphic clathrates of cephalosporin: cefazolin sodium pentahydrate (alpha form) and FK041 hydrate. *Chemical & pharmaceutical bulletin* **50**: 766-770.

Mireles JR, Toguchi A, Harshey RM (2001). *Salmonella enterica* Serovar Typhimurium Swarming Mutants with Altered Biofilm-Forming Abilities: Surfactin Inhibits Biofilm Formation. *Journal of Bacteriology* **183**: 5848-5854.

Mockus L, Peterson JJ, Lainez JM, Reklaitis GV (2015). Batch-to-Batch Variation: A Key Component for Modeling Chemical Manufacturing Processes. *Organic Process Research & Development* **19**: 908-914.

Moeller A, Horak F, Lane C, Knight D, Kicic A, Brennan S *et al* (2006). Inducible NO synthase expression is low in airway epithelium from young children with cystic fibrosis. *Thorax* **61**: 514-520.

Moilanen E, Vapaatalo H (1995). Nitric oxide in inflammation and immune response. *Annals of medicine* **27**: 359-367.

Moncada S, Palmer RM, Higgs EA (1991a). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**: 109.

Moncada S, Palmer RM, Higgs EA (1991b). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**: 109-142.

- Monds RD, Newell PD, Gross RH, O'Toole GA (2007). Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0-1 biofilm formation by controlling secretion of the adhesin LapA. *Molecular microbiology* **63**: 656-679.
- Morgan R, Kohn S, Hwang S-H, Hassett DJ, Sauer K (2006). BdlA, a Chemotaxis Regulator Essential for Biofilm Dispersion in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **188**: 7335-7343.
- Morley D, Keefer LK (1993). Nitric oxide/nucleophile complexes: a unique class of nitric oxide-based vasodilators. *Journal of cardiovascular pharmacology* **22 Suppl 7**: S3-9.
- Morrison AJ, Jr., Wenzel RP (1984). Epidemiology of infections due to *Pseudomonas aeruginosa*. *Reviews of infectious diseases* **6 Suppl 3**: S627-642.
- Mouton JW, Muller AE, Canton R, Giske CG, Kahlmeter G, Turnidge J (2017). MIC-based dose adjustment: facts and fables. *Journal of Antimicrobial Chemotherapy*: dkx427-dkx427.
- Mulcahy H, Charron-Mazenod L, Lewenza S (2008). Extracellular DNA Chelates Cations and Induces Antibiotic Resistance in *Pseudomonas aeruginosa* Biofilms. *PLOS Pathogens* **4**: e1000213.
- Murray HD, Schneider DA, Gourse RL (2003). Control of rRNA Expression by Small Molecules Is Dynamic and Nonredundant. *Molecular Cell* **12**: 125-134.
- Nair HAS, Periasamy S, Liang Y, Kjelleberg S, Rice SA (2016). Real Time, Spatial and Temporal Mapping of the Distribution of c-di-GMP During Biofilm Development. *Journal of Biological Chemistry*.
- Nair HAS, Periasamy S, Yang L, Kjelleberg S, Rice SA (2017). Real Time, Spatial, and Temporal Mapping of the Distribution of c-di-GMP during Biofilm Development. *Journal of Biological Chemistry* **292**: 477-487.
- Navarro MVAS, Newell PD, Krasteva PV, Chatterjee D, Madden DR, O'Toole GA *et al* (2011). Structural Basis for c-di-GMP-Mediated Inside-Out Signaling Controlling Periplasmic Proteolysis. *PLoS Biology* **9**: e1000588.

- Nealson KH, Platt T, Hastings JW (1970). Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System. *Journal of Bacteriology* **104**: 313-322.
- Neut D, Hendriks JG, van Horn JR, van der Mei HC, Busscher HJ (2005). *Pseudomonas aeruginosa* biofilm formation and slime excretion on antibiotic-loaded bone cement. *Acta orthopaedica* **76**: 109-114.
- Newell PD, Monds RD, O'Toole GA (2009). LapD is a bis-(3',5')-cyclic dimeric GMP-binding protein that regulates surface attachment by *Pseudomonas fluorescens* Pf0-1. *Proceedings of the National Academy of Sciences* **106**: 3461-3466.
- Newell PD, Boyd CD, Sondermann H, O'Toole GA (2011). A c-di-GMP Effector System Controls Cell Adhesion by Inside-Out Signaling and Surface Protein Cleavage. *PLoS Biology* **9**: e1000587.
- Nguyen T-K, Duong HTT, Selvanayagam R, Boyer C, Barraud N (2015). Iron oxide nanoparticle-mediated hyperthermia stimulates dispersal in bacterial biofilms and enhances antibiotic efficacy. *Scientific Reports* **5**: 18385.
- Nichols WW, Evans MJ, Slack MP, Walmsley HL (1989). The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. *Journal of general microbiology* **135**: 1291-1303.
- Niedbala W, Wei XQ, Piedrafita D, Xu D, Liew FY (1999). Effects of nitric oxide on the induction and differentiation of Th1 cells. *European journal of immunology* **29**: 2498-2505.
- Nijland R, Hall MJ, Burgess JG (2010). Dispersal of Biofilms by Secreted, Matrix Degrading, Bacterial DNase. *PLOS ONE* **5**: e15668.
- Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML (2002). GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. *Molecular microbiology* **45**: 1277-1287.
- Oglesby AG, Farrow JM, Lee J-H, Tomaras AP, Greenberg EP, Pesci EC *et al* (2008a). The Influence of Iron on *Pseudomonas aeruginosa* Physiology: A

REGULATORY LINK BETWEEN IRON AND QUORUM SENSING. *The Journal of Biological Chemistry* **283**: 15558-15567.

Oglesby LL, Jain S, Ohman DE (2008b). Membrane topology and roles of *Pseudomonas aeruginosa* Alg8 and Alg44 in alginate polymerization. *Microbiology (Reading, England)* **154**: 1605-1615.

Oh E, Jeon B (2014). Role of Alkyl Hydroperoxide Reductase (AhpC) in the Biofilm Formation of *Campylobacter jejuni*. *PLoS ONE* **9**: e87312.

Opatowski L, Mandel J, Varon E, Boëlle P-Y, Temime L, Guillemot D (2010). Antibiotic Dose Impact on Resistance Selection in the Community: a Mathematical Model of β -Lactams and *Streptococcus pneumoniae* Dynamics. *Antimicrobial Agents and Chemotherapy* **54**: 2330-2337.

Pamp SJ, Tolker-Nielsen T (2007). Multiple Roles of Biosurfactants in Structural Biofilm Development by *Pseudomonas aeruginosa*. *Journal of Bacteriology* **189**: 2531-2539.

Parsek MR, Singh PK (2003). Bacterial biofilms: an emerging link to disease pathogenesis. *Annual review of microbiology* **57**: 677-701.

Pearson JP, Passador L, Iglewski BH, Greenberg EP (1995). A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 1490-1494.

Pecharki D, Petersen FC, Scheie AA (2008). Role of hyaluronidase in *Streptococcus intermedius* biofilm. *Microbiology (Reading, England)* **154**: 932-938.

Peng HB, Spiecker M, Liao JK (1998). Inducible nitric oxide: an autoregulatory feedback inhibitor of vascular inflammation. *J Immunol* **161**: 1970-1976.

Pesci EC, Milbank JBJ, Pearson JP, McKnight S, Kende AS, Greenberg EP *et al* (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 11229-11234.

- Pessi G, Haas D (2000). Transcriptional Control of the Hydrogen Cyanide Biosynthetic Genes hcnABC by the Anaerobic Regulator ANR and the Quorum-Sensing Regulators LasR and RhIR in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **182**: 6940-6949.
- Petrova OE, Sauer K (2012a). Dispersion by *Pseudomonas aeruginosa* requires an unusual posttranslational modification of BdlA. *Proceedings of the National Academy of Sciences* **109**: 16690-16695.
- Petrova OE, Sauer K (2012b). PAS Domain Residues and Prosthetic Group Involved in BdlA-Dependent Dispersion Response by *Pseudomonas aeruginosa* Biofilms. *Journal of Bacteriology* **194**: 5817-5828.
- Petrova OE, Cherny KE, Sauer K (2015). The Diguanylate Cyclase GcbA Facilitates *Pseudomonas aeruginosa* Biofilm Dispersion by Activating BdlA. *Journal of Bacteriology* **197**: 174-187.
- Pitt WG, Ross SA (2003). ULTRASOUND INCREASES THE RATE OF BACTERIAL CELL GROWTH. *Biotechnol Prog* **19**: 1038-1044.
- Plate L, Marletta MA (2012a). Nitric oxide modulates bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network. *Mol Cell* **46**: 449-460.
- Plate L, Marletta MA (2012b). Nitric oxide modulates bacterial biofilm formation through a multi-component cyclic-di-GMP signaling network. *Molecular Cell* **46**: 449-460.
- Ponnusamy K, Paul D, Kim YS, Kweon JH (2010). 2(5H)-Furanone: a prospective strategy for biofouling-control in membrane biofilm bacteria by quorum sensing inhibition. *Brazilian Journal of Microbiology* **41**: 227-234.
- Poole K, McKay GA (2003). Iron acquisition and its control in *Pseudomonas aeruginosa*: many roads lead to Rome. *Frontiers in bioscience : a journal and virtual library* **8**: d661-686.
- Poschet JF, Fazio JA, Timmins GS, Ornatowski W, Perkett E, Delgado M *et al* (2006). Endosomal hyperacidification in cystic fibrosis is due to defective nitric oxide-cyclic GMP signalling cascade. *EMBO Reports* **7**: 553-559.

- Purevdorj-Gage B, Costerton WJ, Stoodley P (2005). Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. *Microbiology (Reading, England)* **151**: 1569-1576.
- Ramamurthi A, Lewis RS (1997). Measurement and Modeling of Nitric Oxide Release Rates for Nitric Oxide Donors. *Chemical Research in Toxicology* **10**: 408-413.
- Rao F, Ji Q, Soehano I, Liang Z-X (2011). Unusual Heme-Binding PAS Domain from YybT Family Proteins. *Journal of Bacteriology* **193**: 1543-1551.
- Rayamajhi M, Miao EA (2013). Just say NO to NLRP3. *Nature immunology* **14**: 12-14.
- Rebbapragada A, Johnson MS, Harding GP, Zuccarelli AJ, Fletcher HM, Zhulin IB *et al* (1997). The Aer protein and the serine chemoreceptor Tsr independently sense intracellular energy levels and transduce oxygen, redox, and energy signals for *Escherichia coli* behavior. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 10541-10546.
- Reding-Roman C, Hewlett M, Duxbury S, Gori F, Gudelj I, Beardmore R (2017). The unconstrained evolution of fast and efficient antibiotic-resistant bacterial genomes. *Nature Ecology & Evolution* **1**: 0050.
- Reeve CA, Amy PS, Martin A (1984). Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *Journal of Bacteriology* **160**: 1041-1046.
- Rice SA, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S (2005). Biofilm Formation and Sloughing in *Serratia marcescens* Are Controlled by Quorum Sensing and Nutrient Cues. *Journal of Bacteriology* **187**: 3477-3485.
- Rice SA, Tan CH, Mikkelsen PJ, Kung V, Woo J, Tay M *et al* (2008). The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *ISME J* **3**: 271-282.
- Richards MJ, Edwards JR, Culver DH, Gaynes RP (1999). Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Critical care medicine* **27**: 887-892.

Roberts AP, Mullany P (2010). Oral biofilms: a reservoir of transferable, bacterial, antimicrobial resistance. *Expert review of anti-infective therapy* **8**: 1441-1450.

Robinson JL, Brynildsen MP (2016). Discovery and dissection of metabolic oscillations in the microaerobic nitric oxide response network of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **113**: E1757-E1766.

Rodriguez-Martinez JM, Poirel L, Nordmann P (2009). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **53**: 4783-4788.

Rodriguez-Rojas A, Oliver A, Blazquez J (2012). Intrinsic and environmental mutagenesis drive diversification and persistence of *Pseudomonas aeruginosa* in chronic lung infections. *The Journal of infectious diseases* **205**: 121-127.

Römling U, Galperin MY, Gomelsky M (2013). Cyclic di-GMP: the First 25 Years of a Universal Bacterial Second Messenger. *Microbiology and Molecular Biology Reviews : MMBR* **77**: 1-52.

Rosano GL, Ceccarelli EA (2014). Recombinant protein expression in *Escherichia coli*: advances and challenges. *Frontiers in Microbiology* **5**: 172.

Rowe MC, Withers HL, Swift S (2010a). Uropathogenic *Escherichia coli* forms biofilm aggregates under iron restriction that disperse upon the supply of iron. *FEMS Microbiol Lett* **307**: 102-109.

Rowe MC, Withers HL, Swift S (2010b). Uropathogenic *Escherichia coli* forms biofilm aggregates under iron restriction that disperse upon the supply of iron. *FEMS Microbiol Lett* **307**: 102-109.

Roy A, Srinivasan N, Gowri VS (2009). Molecular and structural basis of drift in the functions of closely-related homologous enzyme domains: implications for function annotation based on homology searches and structural genomics. *In silico biology* **9**: S41-55.

Roy A, Kucukural A, Zhang Y (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nature protocols* **5**: 725-738.

- Roy AB, Petrova OE, Sauer K (2012). The Phosphodiesterase DipA (PA5017) Is Essential for *Pseudomonas aeruginosa* Biofilm Dispersion. *Journal of Bacteriology* **194**: 2904-2915.
- Rybtke M, Berthelsen J, Yang L, Høiby N, Givskov M, Tolker-Nielsen T (2015). The LapG protein plays a role in *Pseudomonas aeruginosa* biofilm formation by controlling the presence of the CdrA adhesin on the cell surface. *MicrobiologyOpen* **4**: 917-930.
- Saavedra JE, Booth MN, Hrabie JA, Davies KM, Keefer LK (1999). Piperazine as a Linker for Incorporating the Nitric Oxide-Releasing Diazeniumdiolate Group into Other Biomedically Relevant Functional Molecules. *The Journal of Organic Chemistry* **64**: 5124-5131.
- Sakuragi Y, Kolter R (2007). Quorum-Sensing Regulation of the Biofilm Matrix Genes (pel) of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **189**: 5383-5386.
- Sasnow SS, Wei H, Aristilde L (2016). Bypasses in intracellular glucose metabolism in iron-limited *Pseudomonas putida*. *MicrobiologyOpen* **5**: 3-20.
- Sati SP, Singh SK, Kumar N, Sharma A (2002). Extra terminal residues have a profound effect on the folding and solubility of a *Plasmodium falciparum* sexual stage-specific protein over-expressed in *Escherichia coli*. *European Journal of Biochemistry* **269**: 5259-5263.
- Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002). *Pseudomonas aeruginosa* Displays Multiple Phenotypes during Development as a Biofilm. *Journal of Bacteriology* **184**: 1140-1154.
- Sauer K, Cullen MC, Rickard AH, Zeef LA, Davies DG, Gilbert P (2004). Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* **186**: 7312-7326.
- Savitsky P, Bray J, Cooper CDO, Marsden BD, Mahajan P, Burgess-Brown NA *et al* (2010). High-throughput production of human proteins for crystallization: The SGC experience. *Journal of Structural Biology* **172**: 3-13.
- Schairer DO, Chouake JS, Nosanchuk JD, Friedman AJ (2012). The potential of nitric oxide releasing therapies as antimicrobial agents. *Virulence* **3**: 271-279.

Schertzer JW, Brown SA, Whiteley M (2010). Oxygen levels rapidly modulate *Pseudomonas aeruginosa* social behaviours via substrate limitation of PqsH. *Molecular microbiology* **77**: 1527-1538.

Schleheck D, Barraud N, Klebensberger J, Webb JS, McDougald D, Rice SA *et al* (2009). *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. *PLoS One* **4**: e5513.

Schmidt I, Steenbakkers PJ, op den Camp HJ, Schmidt K, Jetten MS (2004). Physiologic and proteomic evidence for a role of nitric oxide in biofilm formation by *Nitrosomonas europaea* and other ammonia oxidizers. *J Bacteriol* **186**: 2781-2788.

Schneider DA, Gaal T, Gourse RL (2002). NTP-sensing by rRNA promoters in *Escherichia coli* is direct. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 8602-8607.

Schneider DA, Gourse RL (2004). Relationship between Growth Rate and ATP Concentration in *Escherichia coli*: A BIOASSAY FOR AVAILABLE CELLULAR ATP. *Journal of Biological Chemistry* **279**: 8262-8268.

Schonafinger K (1999). Heterocyclic NO prodrugs. *Farmaco (Societa chimica italiana : 1989)* **54**: 316-320.

Schönafinger K (1999). Heterocyclic NO prodrugs. *Il Farmaco* **54**: 316-320.

Schreiber K, Krieger R, Benkert B, Eschbach M, Arai H, Schobert M *et al* (2007). The Anaerobic Regulatory Network Required for *Pseudomonas aeruginosa* Nitrate Respiration. *Journal of Bacteriology* **189**: 4310-4314.

Shepard BD, Gilmore MS (2002). Differential expression of virulence-related genes in *Enterococcus faecalis* in response to biological cues in serum and urine. *Infect Immun* **70**: 4344-4352.

Sheremetev AB, Makhova NN, Friedrichsen W (2001). Monocyclic furazans and furoxans. *Advances in Heterocyclic Chemistry*. Academic Press. pp 65-188.

Shoda LK, Kegerreis KA, Suarez CE, Roditi I, Corral RS, Bertot GM *et al* (2001). DNA from protozoan parasites *Babesia bovis*, *Trypanosoma cruzi*, and *T. brucei*

is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. *Infect Immun* **69**: 2162-2171.

Simm R, Morr M, Kader A, Nimtz M, Romling U (2004). GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Molecular microbiology* **53**: 1123-1134.

Singh RJ, Hogg N, Joseph J, Kalyanaraman B (1996). Mechanism of nitric oxide release from S-nitrosothiols. *J Biol Chem* **271**: 18596-18603.

Sorba G, Medana C, Fruttero R, Cena C, Di Stilo A, Galli U *et al* (1997). Water Soluble Furoxan Derivatives as NO Prodrugs. *Journal of Medicinal Chemistry* **40**: 463-469.

Stevanin TM, Ioannidis N, Mills CE, Kim SO, Hughes MN, Poole RK (2000). Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes bo' or bd, from nitric oxide. *J Biol Chem* **275**: 35868-35875.

Stewart GS, Williams P (1992). *lux* genes and the applications of bacterial bioluminescence. *Journal of general microbiology* **138**: 1289-1300.

Stewart PS, Costerton WJ (2001). Antibiotic resistance of bacteria in biofilms. *The Lancet* **358**: 135-138.

Stoodley P, Wilson S, Hall-Stoodley L, Boyle JD, Lappin-Scott HM, Costerton JW (2001). Growth and Detachment of Cell Clusters from Mature Mixed-Species Biofilms. *Applied and Environmental Microbiology* **67**: 5608-5613.

Stoodley P, Sauer K, Davies DG, Costerton JW (2002). Biofilms as complex differentiated communities. *Annual review of microbiology* **56**: 187-209.

Suk SE, Yoo JS, Lee JK, Lee KM, Chung GT, Shin ES *et al* (2007). Investigation of beta-lactamase-producing multidrug-resistant *Pseudomonas aeruginosa* isolated from non-tertiary care hospitals in Korea. *Journal of microbiology and biotechnology* **17**: 1733-1737.

Sulemankhil I, Ganopolsky JG, Dieni CA, Dan AF, Jones ML, Prakash S (2012). Prevention and Treatment of Virulent Bacterial Biofilms with an Enzymatic

Nitric Oxide-Releasing Dressing. *Antimicrobial Agents and Chemotherapy* **56**: 6095-6103.

Sumantran VN (2011). Cellular chemosensitivity assays: an overview. *Methods in molecular biology (Clifton, NJ)* **731**: 219-236.

Tacconelli E, Magrini N (2017). Global priority list of antibiotic-resistant bacteria to guide resesarch, discovery and development of new antibiotics. *World Health Organization*.

Tan SY-Y, Liu Y, Chua SL, Vejborg RM, Jakobsen TH, Chew SC *et al* (2014). Comparative Systems Biology Analysis To Study the Mode of Action of the Isothiocyanate Compound Iberin on *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **58**: 6648-6659.

Taylor BL, Zhulin IB (1999). PAS Domains: Internal Sensors of Oxygen, Redox Potential, and Light. *Microbiology and Molecular Biology Reviews* **63**: 479-506.

Thomas DD, Espey MG, Ridnour LA, Hofseth LJ, Mancardi D, Harris CC *et al* (2004). Hypoxic inducible factor 1 α , extracellular signal-regulated kinase, and p53 are regulated by distinct threshold concentrations of nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 8894-8899.

Thomas DD, Ridnour L, Donzelli S, Espey MG, Mancardi D, Isenberg JS *et al* (2006). The Chemistry of Protein Modifications Elicited by Nitric Oxide and Related Nitrogen Oxides. *Redox Proteomics*. John Wiley & Sons, Inc. pp 25-58.

Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S *et al* (2008a). The chemical biology of nitric oxide: Implications in cellular signaling. *Free Radical Biology and Medicine* **45**: 18-31.

Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzellie S *et al* (2008b). The Chemical Biology of Nitric Oxide. Implications in Cellular Signaling. *Free radical biology & medicine* **45**: 18-31.

Thomas SR, Kharitonov SA, Scott SF, Hodson ME, Barnes PJ (2000). Nasal and exhaled nitric oxide is reduced in adult patients with cystic fibrosis and does not correlate with cystic fibrosis genotype. *Chest* **117**: 1085-1089.

- Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH *et al* (2011). *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation *in vivo*. *Journal of immunology (Baltimore, Md : 1950)* **186**: 6585-6596.
- Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS, Molin S (2000). Development and Dynamics of *Pseudomonas* sp. Biofilms. *Journal of Bacteriology* **182**: 6482-6489.
- Toyofuku M, Nomura N, Fujii T, Takaya N, Maseda H, Sawada I *et al* (2007). Quorum Sensing Regulates Denitrification in *Pseudomonas aeruginosa* PAO1. *Journal of Bacteriology* **189**: 4969-4972.
- Toyofuku M, Nomura N, Kuno E, Tashiro Y, Nakajima T, Uchiyama H (2008). Influence of the *Pseudomonas* Quinolone Signal on Denitrification in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **190**: 7947-7956.
- Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM *et al* (2013). Daptomycin-Resistant *Enterococcus faecalis* Diverts the Antibiotic Molecule from the Division Septum and Remodels Cell Membrane Phospholipids. *mBio* **4**.
- Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR *et al* (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature protocols* **7**: 562-578.
- Trunk K, Benkert B, Quack N, Munch R, Scheer M, Garbe J *et al* (2010). Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. *Environ Microbiol* **12**: 1719-1733.
- Umansky V, Hehner SP, Dumont A, Hofmann TG, Schirmacher V, Droge W *et al* (1998). Co-stimulatory effect of nitric oxide on endothelial NF-kappaB implies a physiological self-amplifying mechanism. *European journal of immunology* **28**: 2276-2282.
- Uppuluri P, Chaturvedi AK, Srinivasan A, Banerjee M, Ramasubramaniam AK, Köhler JR *et al* (2010). Dispersion as an Important Step in the *Candida albicans* Biofilm Developmental Cycle. *PLoS Pathogens* **6**: e1000828.

- Valentini M, Filloux A (2016). Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. *The Journal of Biological Chemistry* **291**: 12547-12555.
- van Ogtrop ML, Mattie H, Guiot HF, van Strijen E, Hazekamp-van Dokkum AM, van Furth R (1990). Comparative study of the effects of four cephalosporins against *Escherichia coli* *in vitro* and *in vivo*. *Antimicrobial Agents and Chemotherapy* **34**: 1932-1937.
- Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003). Contributions of Antibiotic Penetration, Oxygen Limitation, and Low Metabolic Activity to Tolerance of *Pseudomonas aeruginosa* Biofilms to Ciprofloxacin and Tobramycin. *Antimicrobial Agents and Chemotherapy* **47**: 317-323.
- Warren JB, Loi R, Rendell NB, Taylor GW (1990). Nitric oxide is inactivated by the bacterial pigment pyocyanin. *The Biochemical journal* **266**: 921-923.
- Watts KJ, Taylor BL, Johnson MS (2011). PAS/Poly-HAMP Signalling in AER-2, a soluble heme-based sensor. *Molecular microbiology* **79**: 686-699.
- Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B *et al* (2003). Cell Death in *Pseudomonas aeruginosa* Biofilm Development. *Journal of Bacteriology* **185**: 4585-4592.
- Webert KE, Vanderzwan J, Duggan M, Scott JA, McCormack DG, Lewis JF *et al* (2000). Effects of inhaled nitric oxide in a rat model of *Pseudomonas aeruginosa* pneumonia. *Critical care medicine* **28**: 2397-2405.
- Whitney JC, Colvin KM, Marmont LS, Robinson H, Parsek MR, Howell PL (2012). Structure of the Cytoplasmic Region of PelD, a Degenerate Diguanylate Cyclase Receptor That Regulates Exopolysaccharide Production in *Pseudomonas aeruginosa*. *The Journal of Biological Chemistry* **287**: 23582-23593.
- WHO (2015). WHO Model List of Essential Medicines, 19th List (April 2015).
- Wilson S, Hamilton MA, Hamilton GC, Schumann MR, Stoodley P (2004). Statistical Quantification of Detachment Rates and Size Distributions of Cell Clumps from Wild-Type (PAO1) and Cell Signaling Mutant (JP1) *Pseudomonas aeruginosa* Biofilms. *Applied and Environmental Microbiology* **70**: 5847-5852.

- Witte MB, Barbul A (2002). Role of nitric oxide in wound repair. *American journal of surgery* **183**: 406-412.
- Wolter DJ, Lister PD (2013). Mechanisms of beta-lactam resistance among *Pseudomonas aeruginosa*. *Current pharmaceutical design* **19**: 209-222.
- Wolz C, Hohloch K, Ocaktan A, Poole K, Evans RW, Rochel N *et al* (1994). Iron release from transferrin by pyoverdinin and elastase from *Pseudomonas aeruginosa*. *Infection and Immunity* **62**: 4021-4027.
- Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC *et al* (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *The Journal of clinical investigation* **109**: 317-325.
- Yam JKH, Aung TT, Chua SL, Cheng Y, Kohli GS, Zhou J *et al* (2017). Cyclic-di-GMP is required for corneal infection by *Pseudomonas aeruginosa* and modulates host immunity. *bioRxiv*.
- Yarwood JM, Bartels DJ, Volper EM, Greenberg EP (2004). Quorum Sensing in *Staphylococcus aureus* Biofilms. *Journal of Bacteriology* **186**: 1838-1850.
- Ye RW, Averill BA, Tiedje JM (1994). Denitrification: production and consumption of nitric oxide. *Applied and Environmental Microbiology* **60**: 1053-1058.
- Yepuri NR, Barraud N, Mohammadi NS, Kardak BG, Kjelleberg S, Rice SA *et al* (2013). Synthesis of cephalosporin-3'-diazeniumdiolates: biofilm dispersing NO-donor prodrugs activated by beta-lactamase. *Chemical communications (Cambridge, England)* **49**: 4791-4793.
- Yoon MY, Lee K-M, Park Y, Yoon SS (2011). Contribution of Cell Elongation to the Biofilm Formation of *Pseudomonas aeruginosa* during Anaerobic Respiration. *PLOS ONE* **6**: e16105.
- Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC *et al* (2002). *Pseudomonas aeruginosa* Anaerobic Respiration in Biofilms. *Developmental Cell* **3**: 593-603.

Zeng X, Lin J (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Front Microbiol* **4**: 128.

Zhang L (2011). *Heme Biology: The Secret Life of Heme in Regulating Diverse Biological Processes*. World Scientific.

Zhang W, Prausnitz MR, Edwards A (2004). Model of transient drug diffusion across cornea. *Journal of Controlled Release* **99**: 241-258.

Zhu X, Oh H-S, Ng YCB, Tang PYP, Barraud N, Rice SA (2017). Nitric oxide-mediated induction of dispersal in *Pseudomonas aeruginosa* biofilms is inhibited by flavohemoglobin production and is enhanced by imidazole. *Antimicrobial Agents and Chemotherapy*.