An in-vitro evaluation of C-di-GMP mediated biofilm dispersal as a potential strategy to restore antimicrobial efficacy

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**AN *IN-VITRO* EVALUATION OF C-DI-GMP MEDIATED
BIOFILM DISPERSAL AS A POTENTIAL STRATEGY TO
RESTORE ANTIMICROBIAL EFFICACY**

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**Interdisciplinary Graduate School
Singapore Centre for
Environmental Life Sciences Engineering**

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Doctor of Philosophy

2018

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Summary

The World Health Organization (WHO) has deemed antimicrobial resistance (AMR) in bacteria to be a critical threat to society (World Health Organization 2016, World Health Organization 2017). AMR and specifically multi-drug resistant (MDR) bacteria represent an increasingly serious public health concern worldwide. The need for novel therapeutic approaches to prevent and/or eradicate microbial infections is urgent. AMR can evolve due to mutations that reduce antibiotic susceptibility or due to activation of various innate mechanisms of tolerance, for example, biofilm formation. Bacteria exhibit a complex life-cycle, whereby they can switch from a planktonic, free-living form, to a densely packed biofilm form. Biofilms are an aggregated, sessile, often surface attached form of bacterial mode of growth. Biofilms provide complex 3D structures to bacterial communities, allowing them to interact with each other and respond to changes in their environment differently than their planktonic counterparts (Costerton, Geesey et al. 1978). 60-80% of infections are biofilm-related, once biofilms attach and mature bacteria can exhibit 10 to 1000 times less susceptibility to antimicrobials (Høiby, Bjarnsholt et al. 2010).

The use of dispersal agents has been suggested as a potential treatment strategy against microbial biofilms (Banin, Brady et al. 2006, Frederiksen, Pressler et al. 2006, Landini, Antoniani et al. 2010, Barraud, J. Kelso et al. 2015). This strategy consists of manipulating biofilm dispersal to induce changes in biofilm matrix and metabolic states of biofilm cells. These changes are believed to enhance antimicrobial efficacy, increasing antimicrobial killing

efficiency, shortening duration of treatment and reducing the risk of emergence of resistance. Investigating the effectiveness of biofilm dispersal as part of an antimicrobial treatment strategy is challenging, and the consequences and the underlying mechanisms of such strategy remain unclear. So far, no dispersal agents have yet been used extensively in a clinical setting (Fleming and Rumbaugh 2017). The present research aims at providing knowledge about how c-di-GMP mediated biofilm dispersal will impact antimicrobial synergy treatment against bacterial biofilms and whether it will result in the emergence of resistant/tolerant variant populations.

To achieve these objectives, a strain of *Pseudomonas aeruginosa*, PAO1/p_{BAD}-*yhjH* which carries an inducible phosphodiesterase (PDE) enzyme allowing for controlled biofilm dispersal, was utilized (Chua, Tan et al. 2013). A strain PAO1/pJN105 carrying an empty vector plasmid was used as a control. Using antimicrobial agents, alone or in combination, under static *in-vitro* conditions, c-di-GMP mediated biofilm dispersal was shown to enhance drug synergy against *P. aeruginosa* biofilms (Chapter 2), without increasing the risk of releasing resistant/tolerant variants (Chapter 3). The potential of biofilm dispersal strategy was also assessed against an evolved AMR strain (Chapter 4). A colistin-resistant derivative of PAO1/p_{BAD}-*yhjH* was generated and characterized: Significant differences in morphology, motility, and virulence factor production were identified between the parent PAO1/p_{BAD}-*yhjH* strain and its colistin resistant derivative. Whole genome sequencing and analysis revealed point mutations in *phoQ* and *lpxC*, two genes involved in the biosynthesis of lipid-A located in the bacterial membranes and the biological target of colistin. These mutations resulted in upregulated expression of the

arn operon which is known to lead to the modification of Lipid-A. The colistin-resistant derivative also showed enhanced susceptibility to carbapenems and glycopeptides, suggesting that the development of colistin resistance resulted in a trade-off in sensitivity to other classes of antimicrobials. This was further explored by testing biofilms for sensitivity to a range of antimicrobial combinations. It was observed that colistin plus vancomycin demonstrated a synergistic effect only against the dispersed biofilms of the colistin resistant strain. The results of this work indicate the potential of c-di-GMP mediated biofilm dispersal as a potential strategy for efficient treatment of biofilm-associated infections.

Author's Publications

Journal Publications

The-Thien Tran, Celine Vidaillac, Hong Yu, Valerie F. L. Yong, **Dan Roizman**, Ravishankar Chandrasekaran, Albert Y. H. Lim, Teck Boon Low, Gan Liang Tan, John A. Abisheganaden, Mariko Siyue Koh, Jeanette Teo, Sanjay Haresh Chotrimall, Kunn Hadinoto Ong. (2018). "A new therapeutic avenue for bronchiectasis: Dry powder inhaler of ciprofloxacin nanoplex exhibits superior ex-vivo mucus permeability and antibacterial efficacy to its native ciprofloxacin counterpart". International Journal of Pharmaceutics. 2018 Aug, 547 (1-2), 368-376.

Dan Roizman, Celine Vidaillac, Michael Givskov, Liang Yang. (2017). "*In-vitro* evaluation of biofilm dispersal as a therapeutic strategy to restore antimicrobial efficacy". Antimicrobial Agents & Chemotherapy. 2017 Oct, 61(10) e01088-17.

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Martin Tay, **Dan Roizman**, Yehuda Cohen, Tim Tolker-Nielsen, Michael Givskov, Yang Liang. (2014). "Draft genome sequence of the model naphthalene-utilizing organism *Pseudomonas putida* OUS82". Genome Announcements. 2014 Feb, 2(1) e01161-13.

Poster Presentations

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Abbreviations

<i>A. baumannii</i>	<i>Acinetobacter baumannii</i>
AHL	acylhomoserine lactone
AI	autoinducers
AMR	antimicrobial resistance
A.U	arbitrary units
AUC	area under curve
°C	degrees Celsius
C _{max}	maximum serum concentration
CBD	Calgary Biofilm Pin Lid Device
CDC	Center for Disease Control and Prevention
c-di-GMP	3',5'-Cyclic diguanylic acid
CF	Cystic Fibrosis
CFU	colony forming units
CV	Crystal Violet
CLSI	Clinical Standards Institute
CLSM	confocal laser scanning microscopy
DGC	diguanylate cyclase
DTU	Technical University of Denmark
<i>E. coli</i>	<i>Escherichia coli</i>
eDNA	extracellular DNA
EPS	extracellular polymeric substances
FBEC	fractional biofilm eradication concentration
FIC	fractional inhibitory concentration
g	gram
GFP	Green Fluorescent Protein
Gm ^r	Gm resistance
k	kilo (10 ³)
kHz	kilohertz (1000 cycles per second)

<i>K. pneumonia</i>	<i>Klebsiella pneumonia</i>
L	liter
LB	Lysogeny Broth
LPS	lipopolysaccharides
μ	micro (10^{-6})
m	meter
m	milli (10^{-3})
M	molar (mol per liter)
MDR	multi-drug resistance
MIC	minimal inhibitory concentration
MHB	Mueller-Hinton Broth II
MHA	Mueller-Hinton Agar II
MBC	minimal bactericidal concentration
MBIC	minimal biofilm inhibitory concentration
MBEC	minimal biofilm eradication concentration
MDK	minimal duration for killing
MNV	Multi-nucleotide Variation
mol	mole (amount of substance)
mw	molecular weight
n	nano (10^{-9})
NADH	Nicotinamide-adenine-dinucleotide
O.D	optical density
O.N.	overnight
p	pico (10^{-12})
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PAP	population analysis profile
PBP	penicillin-binding protein
PDE	phosphodiesterase
QS	quorum sensing

REMA	Resazurin Microtiter Assay
rpm	revolutions per minute
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SCV	Small-colony variant
SNV	Single-nucleotide Variation
STDEV	Standard Deviation
UV	ultra violet
vol/vol	volume per volume
WHO	World Health Organization
<i>w.t</i>	wild-type
w/v	weight per volume

Chapter 1: Literature Review

1.1 Antimicrobial Resistance (AMR)

Since the initial discovery of penicillin by Alexander Fleming in the 1920s, antibiotics have been the primary treatment agents for acute and chronic infections caused by bacteria (Huttner, Harbarth et al. 2013). The development of new antibiotics has substantially slowed down in recent decades (World Health Organization 2016). The Review on Antimicrobial Resistance (AMR) commissioned in July 2014 by the United Kingdom (O'Neill 2014) has estimated that in the near future, AMR and microbial infections will surpass cancer in death toll and costs, over 10 million deaths per year and at least \$100 trillion of the British gross national product. Those conclusions were recently supported by the World Health Organization (WHO) who has called for researchers, health care professionals and officials to collaborate and focus on global prevention and stewardship programs (World Health Organization 2016).

The frequent administration and overuse both for human health and agriculture have been associated with the increased spread of AMR over recent decades. It is estimated that the prevalence of acquired hospital infections is in the range of 1-10% of all patients admitted and that this is likely to be higher in developing countries (Allegranzi, Nejad et al. 2010). Estimated costs for AMR related infections in the US alone are in the range of at least 55 billion USD when parameters such as prolonged length of stay, more intensive

antibiotic treatment regimens utilized, additional complications and higher mortality rates are calculated (Smith and Coast 2012).

AMR phenotypes can be divided into three main categories: Resistance, Tolerance, and Persistence (Brauner, Fridman et al. 2016). Resistant bacteria are bacteria that express or acquire new genes that allow them to sustain increasing concentrations of antibiotics; such as enzymes that actively degrade antimicrobials, scaffold proteins that are a “barrier” for antibiotics or develop resistance through mutation of antibiotic targets (specific mechanisms in the next section). Tolerance, on the other hand, allows subpopulations to withstand transient exposures to high antibiotic concentrations. The ability to form biofilms has been linked to increased tolerance of bacteria.

Bacteria exhibit a complex life cycle, switching from planktonic cells to an aggregated, sessile, often surface attached form, called a biofilm, under different conditions. Approximately 60-80% of infections are biofilm-associated and can be categorized into two groups: 1) Medical device-related infections (such as catheters, stents) & 2) infections of host tissue (skin, lungs or heart) (Høiby, Bjarnsholt et al. 2015). These infections are defined as “hard to treat”, usually chronic, and represent a therapeutic challenge for clinicians, especially when they involve multiple species, multidrug-resistant isolates and when the site of infection is difficult to reach (Gellatly and Hancock 2013). In biofilms, the extracellular matrix represents a diffusion barrier to antimicrobials resulting in the delivery of sub-inhibitory concentrations of the antibiotics to the cells. Bacteria also exist in different metabolic states, which ultimately affect their susceptibility to antimicrobial agents. Antimicrobial combination therapy

has been shown to exhibit higher efficacy in treating biofilms (Černohorská and Votava 2008, Herrmann, Yang et al. 2010, Hengzhuang, Wu et al. 2011). Nonetheless, it harbors some limitations such as treatment durations, which can remain long and therefore, favorable for the development of drug resistance or side effects.

Persistence occurs when a small portion (<0.01%) of the bacterial population becomes metabolically dormant (Brauner, Fridman et al. 2016). This random, subpopulation of cells is therefore insensitive to the antibiotics and survives. Persistence is a non-heritable trait, induced by various environmental stresses, such as nutrient depletion. Persistent cells exist as part of the population, were considered until recently as viable but non-culturable, and when the antibiotic stress is removed this cell population regrows as normal cells (Kim, Chowdhury et al. 2018).

1.2 Antibiotic Resistance Mechanisms

As described above, bacteria have a number of strategies that enable them to survive the antibiotic pressure. The opportunistic pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) is an example of such pathogens that is intrinsically resistant to several groups of antimicrobials. This bacterium can augment resistance with tolerance and persistence mechanisms and also can acquire resistance through horizontal gene transfer (Livermore 2002). The following sections present general bacterial resistance mechanisms to different classes of antibiotics, which are widely distributed in *P. aeruginosa*.

Carbapenems

Carbapenem antimicrobials belong to the broader antibiotic group of β -lactams (Zhanel, Wiebe et al. 2007). These compounds have structural similarity to penicillin and cephalosporin antibiotics. All β -lactams are made of a cyclic ring structure motif, and their primary target is the penicillin-binding protein (PBP) transpeptidase enzyme family, which governs the synthesis of the bacterial cell wall (Morita, Tomida et al. 2013). Due to low oral availability, carbapenems are best administered intravenously, and due to their broad-spectrum activity, they are often used to prevent nosocomial infections (Rello 2007). The two carbapenem agents frequently administered to treat *P. aeruginosa* are imipenem and meropenem.

The target sites for carbapenems are adjacent to the bacterial inner membrane, at the intermembrane space, and cytosol (Chen, Zhang et al. 2016). Carbapenems need to pass the selective barrier of the outer cell membrane, through porin channels, and more specifically the complex OprD (Amin, Giske et al. 2005). Variants of *P. aeruginosa* expressing lower levels of this porin exhibit slower growth rates but also become tolerant to carbapenems. *P. aeruginosa*, like many other organisms, can express β -lactamase enzymes, which actively degrade β -lactam antibiotics; these enzymes can have very different modes of action and can exist in several copies in bacteria. Most β -lactamases are inefficient in degrading carbapenems, though continued exposure can lead to overexpression of β -lactamases, especially AmpC, and also mutated enzymes with better capabilities have been reported (Wolter and Lister 2013). Some bacteria express multidrug efflux pumps, such as MexAB-OprM, MexCD-OprJ,

encoded by *P. aeruginosa* (Okamoto, Gotoh et al. 2002). Mutations to the PBPs, targets of the carbapenems, have been shown to be heterogenic, either culminate in mutations to targets themselves or changes in the expression profiles of the genes PBP1-4 depending on isolates (Moyá, Beceiro et al. 2012).

Aminoglycosides

Aminoglycosides are a group of antibiotics that have several sugar motifs bound to amino groups through the anomeric carbons (Forge and Schacht 2000). The targets of these compounds are the 30S small ribosomal units of bacteria, causing inhibition of protein synthesis. The most common aminoglycosides administered for *P. aeruginosa* infections are streptomycin, kanamycin, neomycin, and gentamicin, although tobramycin displays the most significant killing potential of the aminoglycoside antibiotics. The high tolerance of *P. aeruginosa* biofilm to tobramycin has been known since its introduction to treatment (Nickel, Ruseska et al. 1985).

Resistance can be mediated by enzymes that modify the aminoglycosides: aminoglycoside phosphoryl-transferase, aminoglycoside acetyl-transferase, and amino-glycoside nucleotidyltransferase (Poole 2005). The principal mechanism of tolerance employed by *P. aeruginosa* is impermeability and active transport by multidrug efflux pumps such as MexXY-OprM (Westbrock-Wadman, Sherman et al. 1999, Bulitta, Ly et al. 2015). Furthermore, since there is a need for binding with the lipopolysaccharides (LPS) of the outer membrane of the cell before penetration, the additional potential for resistance exists (Schniederjans, Koska et al. 2017). It has been shown that mutations

that lead to alterations of the LPS lead to higher resistance in *P. aeruginosa* (Schurek, Marr et al. 2008, Krahn, Gilmour et al. 2012).

Polymyxins

The polymyxin antibiotic group comprises molecules with a cyclic polypeptide motif, bound to a fatty acid moiety (Storm, Rosenthal et al. 1977), a lipopeptide. The two members of this group that are frequently administered to treat *P. aeruginosa* infections are Polymyxin B and Polymyxin E, the latter commercially known as Colistin. Polymyxins act via binding of LPS by the polypeptide motif and bacterial membrane disruption by the fatty acid chain to cause lysis (Biswas, Brunel et al. 2012). Polymyxins attack bacterial cells regardless of metabolic state (Breidenstein, de la Fuente-Núñez et al. 2011). Thus colistin is potentially effective against non-dividing cells and persister cells (Cui, Niu et al. 2016).

The primary mechanisms of resistance are modifications to the lipid-A lipopolysaccharide, the main constituent of the outer monolayer of the bacterial outer membrane. The PhoP-PhoQ and PmrA-PmrB two-component systems (TCS) have been found to regulate the *arn* operon (Breidenstein, de la Fuente-Núñez et al. 2011). Enzymes encoded in this operon constitute Lipid-A modifications such as additions of 4-amino-L-arabinose or phosphor-ethanolamine (Fernández, Álvarez-Ortega et al. 2013). These changes reduce the overall net negative charge of the membrane, limiting the reaction with polymyxins (Lee, Park et al. 2016). Polymyxins displace Mg^{+2} and Ca^{+2} ions from the LPS membrane, before fully binding to Lipid-A, and their activity depends on the concentration of these divalent ions and pH (Conly and

Johnston 2006). The PhoP-PhoQ system is expressed together in one operon with the protein OprH that binds directly to the LPS and assists in its stabilization (Lim, Ly et al. 2010).

1.3 Biofilm-associated tolerance

It is estimated that about two-thirds of hospital-acquired infections are biofilm-related (Høiby, Bjarnsholt et al. 2010). Biofilms are associated with medical device-related infections as well as chronic infections that are recalcitrant to clearance by the host immune system or antibiotic therapy (Lewis 2008). Indeed, biofilms are associated with a general increase in the survival of bacteria when challenged with extreme environmental conditions, immune system (e.g., reactive oxygen) or antibiotic stresses. For example, it has been reported that 100-1000 fold higher concentrations of antibiotics are required to kill biofilm bacteria compared to planktonic cells (Spoering and Lewis 2001).

The increased tolerance of bacteria within biofilms appears to be multifactorial (Høiby, Bjarnsholt et al. 2010). Bacteria have been shown to be physiologically stratified in biofilms, where cells on the surface tend to be more active while bacteria within the interior may be less active, dormant or undergoing anaerobic metabolism (Williamson, Richards et al. 2012). Thus, the cells in these different states may respond to antibiotics differently, if, for example, the antibiotic mechanism involves attacking metabolically active cells under aerobic conditions (Martínez and Rojo 2011). Biofilm bacteria excrete and form an intricate, heterogeneous but organized matrix composed mainly of polysaccharides, proteins, and extracellular DNA (eDNA) cumulatively termed

“extracellular polymeric substances” (EPS) (Flemming, Wingender et al. 2016). The ratios of “substances” in the EPS differ from species to species and depend on environmental conditions, as well as extracellular queues. Stochastic changes such as threats and stress factors like antibiotics or availability of nutrients and electron acceptors are just some factors that can affect the EPS constituents (Stewart and Franklin 2008).

The EPS has been suggested to lessen penetration of some antibiotics and hence, protects the underlying cells (Yang, Hu et al. 2011). For example, eDNA, which is one of the major EPS components of *P. aeruginosa* biofilms, is negatively charged and thus acts as a passive diffusion barrier to cation antibiotics. Through the chelation of Mg^{2+} and Ca^{2+} overproduction of eDNA has been shown to increase antibiotic resistance through activation of the two-component systems OprH-PhoP/Q and PmrAB (Mulcahy, Charron-Mazenod et al. 2008, Lewenza 2013). In contrast, the exopolysaccharide Psl is neutrally charged polymer composed of repeats of glucose, mannose and rhamnose, and has been suggested to provide a selective barrier to several antibiotic groups (Billings, Ramirez Millan et al. 2013, Flemming, Neu et al. 2016).

Biofilm formation has been shown to select for genetic variants, such as small colony variants (SCV) of *P. aeruginosa*, which were further shown *in-vitro* and *in-vivo* to exhibit higher tolerance to antimicrobials than their ancestors (Kirisits, Prost et al. 2005, Hogardt and Heesemann 2013). SCVs produce large amounts of the exopolysaccharides Psl and Pel while exhibiting reduced motility due to the high intracellular content of c-di-GMP (Starkey, Hickman et al. 2009). The mucoid variants of *P. aeruginosa* were frequently reported to

predominate following infection, and have been shown to overproduce the alginate polysaccharide (Flemming, Neu et al. 2016). The contribution of alginate to antibiotic resistance is still debated, although the reason lies in the fact that it is mostly exhibited by mature biofilms (Govan and Deretic 1996). Collectively, variants are associated with overproduction of EPS constituents, metabolic heterogeneity of the population, which result in higher tolerance and ultimately manifest higher persistence to antibiotics (Starkey, Hickman et al. 2009). SCVs seem to bear all of the mechanisms mentioned above and have been shown to have higher proportions of persister cells when they result from prolonged antibiotic treatment (Wei, Tarighi et al. 2011). **Figure 1.1** symbolizes the multi-factorial recalcitration: heightened antibiotic tolerance, resistance and persistence.

Figure 1.1 A schematic representing tolerance and resistance mechanisms in biofilms, antibiotic penetration (yellow gradient) into the inner levels of biofilm is slowed down due to viscosity and ionic and hydrophobic interactions with EPS constituents (eDNA, Psl). Sub-optimal antibiotic concentration leads to higher expression levels of resistance/tolerance genes in the inner biofilm levels (green);

and heterogenic local environments in biofilms due to oxygen and nutrient gradients (red gradient). All of the above lead to different metabolic rates and heterogenic gene expression. Antibiotic treatment thus results in partial efficacy; not all cells are killed (red bacteria) some live cells always remain (green bacteria). Finally, the biofilm population harbors higher frequency of persister cells (purple bacteria).

The observations that: 1) many infections are associated with biofilms and that 2) biofilm formation facilitates antibiotic resistance and tolerance – therefore, argue that an understanding of how biofilms form is essential. Further, understanding the regulatory mechanisms and key genes in biofilm formation may identify novel targets to better control biofilms by reversing their inherent, high tolerance to antimicrobials. This literature review will provide a general introduction to the biofilm life cycle, focusing on the Gram-negative bacterium that is the model organism used throughout this thesis, *P. aeruginosa*.

1.4 Microbial Life on Surfaces

Biofilm formation is typically described as starting with planktonic, free-living cells migrating to and interacting with surfaces (Flemming, Wingender et al. 2016). Interactions with surfaces as substrata for anchoring are initially unspecific, by means of Van der Waals, electrostatic and hydrophobic interactions (Palmer, Flint et al. 2007). Bacterial surface pili and flagella protein complexes often mediate initial attachment (Klausen, Heydorn et al. 2003). Irreversible attachment is considered the step which is normally cementing the bacteria on the surface, involving repression of the flagellar swimming motility and production of EPS constituents (Ha and O'Toole 2015).

Type IV pili facilitate irreversible binding, and bacterial surface motility, known as twitching (Rasamiravaka, Labtani et al. 2015). An additional form of social motility in biofilms is swarming, which allows for movement of bacteria on semi-solid surfaces and involves flagella, pili, EPS constituents and biosurfactants (Shrout, Chopp et al. 2006). Motility, surface-bound or free-swimming, drives chemotaxis, while still energetically favorable. Once irreversibly attached, biofilms develop by surface-bound growth and further recruitment of cells from the environment, both contribute to the local establishment of micro-colonies (Høiby, Krogh Johansen et al. 2001).

As bacteria grow by multiplication, if the population is sparse there is more room for growth, and it is mostly flat. As the population density increases, physical constraints cause the newly formed bacteria to push towards the periphery (Sheraton, Yam et al. 2018). Microcolony development can result in the formation of a range of different structures depending on the growth conditions, and motility (Ni, Yang et al. 2016). For example, in defined media containing succinate, glutamate or citrate as a sole carbon source, on surfaces which permit swarming, biofilms first appear to develop flat structures (Shrout, Chopp et al. 2006). Biofilm growth and maturation are a function of the available nutrients, physical conditions and cell-to-cell signaling within the biofilm. In continuous culture conditions, elaborate microstructures, resembling mushroom shapes were discovered in *P. aeruginosa* biofilms, on glucose-containing media; with environmental heterogeneity driving the bacteria cells to high differentiation (Garrett, Bhakoo et al. 2008). Both non-motile and motile bacteria contribute to the structure, biofilms continuously aggregate, disperse

and recruit planktonic bacteria from the medium (Klausen, Heydorn et al. 2003).

In a similar manner to planktonic growth kinetics, biofilm's exponential growth persists until nutrients (such as carbon and nitrogen sources) have been depleted from the surrounding environment. Whereas planktonic bacteria acquire nutrients in a "foraging" behavior, biofilm cells are sessile and experience higher metabolic heterogeneity. This heterogeneity in biofilm is due to the impact of neighboring cell metabolism, local internal gradients, nutrient, oxygen or additional electron acceptors or any other related factors within the biofilm. This principle contributes to heterogeneity of differential gene expression between every cell in the biofilm. For example, internal biofilm cells might perform anaerobic fermentation, while external cells in the periphery perform aerobic respiration, and all are sometimes part of the same colony (Stewart and Franklin 2008, Wessel, Arshad et al. 2014). Bacteria in the colony periphery grow more rapidly, versus cells in the center that have stationary growth. Cells near the substratum are attached more firmly, while cells in the outer layers are more freely detached. Recently, a novel *in-silico* biofilm growth development simulation showed that chemotaxis has a role in the shaping of *P. aeruginosa* biofilms, which was confirmed *in-vitro* (Sheraton, Yam et al. 2018). *P. aeruginosa* biofilm development is a constant interplay between the production of different EPS constituents, which are affected by changing environmental flow rates and nutrient availability, different secondary structures such as streamers have been shown to develop under heterogeneous conditions (Nadell, Ricaurte et al. 2017).

It has been established that bacterial cell-to-cell signaling, quorum sensing (QS), is a critical process for the continued development of the biofilm (Miller and Bassler 2001). QS is the coordinated action of the bacterial population, by secretion of small specific signaling molecules, termed autoinducers (AI). As AI concentration increases locally, it cascades in a complete change of the bacterial gene expression, allowing microcolonies to act in unison, at a specific density, the quorum (Li, Li et al. 2015). Three central QS mechanisms that control *P. aeruginosa* biofilm formation are well investigated: *las*, *rhl* and *pqs* (Lee and Zhang 2015). The LasR regulator binds acyl-homoserine-lactone (AHL) activating expression of the operon, the AHL molecule family is a predominant AI in many bacteria (Lade, Paul et al. 2014). In *P. aeruginosa* *las* operon controls a network of reactions, for example, cascading in higher expression of *TpbAB* which negatively controls Pel production and positively controls Psl and eDNA production (Wei and Ma 2013). It was previously shown that a *lasI* mutant of *P. aeruginosa* makes a flat, undifferentiated biofilm that is less stable than the wild-type (*w.t*) biofilm (Sakuragi and Kolter 2007). The *rhl* and *pqs* operons QS systems control the production of the virulence factors rhamnolipid and pyocyanin, respectively; these virulence markers have been shown to be lethal to host immune cells, as well as perturbed epithelial cell tissues that provide a substrate for biofilm formation (Jakobsen, Tolker-Nielsen et al. 2017). QS is associated with the increased tolerance of the biofilm to antimicrobials (Tan, Chua et al. 2013); In recent years there exists a paradigm shift in developing novel antimicrobials, the focus is on the repression of bacterial QS, or AI production (Sintim, Smith et al. 2010). Synthetic AI molecules, QS inhibitors, and antagonists to receptors which take

part in QS are being tested as novel antimicrobials (Beloin, Renard et al. 2014). QS inhibitors of the *las* and *rhl* operons have been shown to impair bacterial motility, virulence, antibiotic resistance and biofilm formation. The use of several antagonists to these QS systems culminates in the protection of the innate immune system and provides potential synergy with antibiotics (Jakobsen, Tolker-Nielsen et al. 2017). These data suggest that interfering with the biofilm developmental program can be exploited to reduce or control infections by *P. aeruginosa*.

1.5 Biofilm dispersal

Mature biofilms complete the life-cycle where they disperse back into free-living planktonic cells. Dispersal has been reported to be induced in response to changing environmental conditions such as oxygen, pH, carbon, and nitrogen, or micronutrient concentrations (Guilhen, Forestier et al. 2017). Bacterial biofilms can be removed due to mechanical forces, or if shear forces are applied, and this process is referred to as sloughing. “Active Biofilm dispersal”, on the other hand, has been found to require energy and is typically mediated by regulatory events upon sensing of changing environmental cues (McDougald, Rice et al. 2011, Fleming and Rumbaugh 2017) (**Figure 1.2**). EPS degrading enzymes, either self-produced or exogenously added have been found to induce dispersal from biofilms. The spatial differentiation of bacteria in biofilms, especially in terms of motility is crucial to the dispersal process and thus, QS systems such as *rhl* and *pqs* also play an active role in the process (Kim and Lee 2016).

Figure 1.2 A scheme representing passive and active dispersal of bacterial biofilms. Passive dispersal (Top) can be caused by external forces such as shearing while active dispersal (Bottom) is the result of different signal transduction cascades, usually involves enzymes that degrade components of the EPS matrix (Figure taken from: (Fleming and Rumbaugh 2017)).

Many of the genetic responses that trigger dispersal are mediated through the secondary messenger, 3',5'-cyclic diguanylic acid (c-di-GMP) (Ha and O'Toole 2015). Secondary messengers relay extracellular cues or signals to the cytoplasmic space, and where catalytic proteins can be activated to synthesize or degrade the intercellular messengers. For many Gram-negative bacteria, the concentration of c-di-GMP controls the mechanism governing the “lifestyle transition” from planktonic to biofilm. Generally, higher intracellular levels of c-di-GMP enhance biofilm formation, while low c-di-GMP levels are associated with dispersal from biofilms or planktonic cells. It is estimated that biofilm bacteria have 75-110 pmol c-di-GMP per mg total protein in the cell extract,

while planktonic bacteria have less than 30 pmol c-di-GMP per mg in the cell extract (Valentini and Filloux 2016). C-di-GMP is produced from GTP by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs).

Dispersed bacteria are distinct in terms of gene expression from biofilm cells and planktonic cells, but following a certain duration of time, they can regain motility, become planktonic, and the cycle begins anew (Chua, Yang et al. 2017). The biofilm life-cycle is usually depicted as a linear development from attachment, to maturation, and then to dispersal, however, it is a cyclic process, with portions of the bacterial cell population existing in all states at the same time in any system, as demonstrated in **figure 1.3**. Most bacteria that form biofilms have the ability to actively disperse them, as a mechanism that allows recolonization in new niches (Guilhen, Forestier et al. 2017).

Figure 1.3 The biofilm life-cycle under positive control by the intracellular c-di-GMP concentration. C-di-GMP is the product of DGC enzyme family and degraded by the

PDE enzyme family. The image shows the correlation between high c-di-GMP (more DGC activity) and biofilm growth and low concentrations (more PDE activity) and dispersal. The *P.aeruginosa* construct used throughout this thesis overexpresses the PDE YhjH when induced, thus facilitating higher levels of biofilm dispersal.

Bacteria have been shown to alter PDE and DGC activity in response to environmental cues to modulate biofilm development. For example, it has been shown that biofilms can be dispersed by exposure to nitric oxide (NO) and that perception of this cue triggers a decrease in intracellular c-di-GMP content, resulting in changes in gene expression associated with the conversion of the biofilm cells into planktonic cells (**Figure 1.4**). Bacteria appear to have evolved the ability to sense and respond to NO. Some bacteria express proteins with PAS domains that bind NO (In *P. aeruginosa* by affecting the activity of chemotaxis regulator *bdlA*), leading to PDE activation, eventually lowering intercellular c-di-GMP concentrations and resulting in biofilm dispersal (Petrova and Sauer 2012).

Figure 1.4 A scheme describing the mechanisms of signal transduction which culminate in changes to the intracellular c-di-GMP concentration that result in differential expression in cells and phenotypic changes (Taken from (McDougald, Rice et al. 2011).

Although not extensively used clinically, combinations of dispersal agents and antibiotics have been proven to facilitate higher synergy *in-vitro* and *in-vivo* (Kaplan 2010). Antibiotic combinations have been proven to be superior in treating biofilm-related infections (Černohorská and Votava 2008, Dundar and Otkun 2010, Barber, Ireland et al. 2014, Chatterjee, Anju et al. 2016), lowering effective concentrations and targeting different cellular mechanisms. Combined antibiotic therapy following facilitated dispersal has not been shown in previous research, though the potential to further improve efficacy at lower concentrations and short treatment durations is a promising option for new therapeutic strategies.

1.6 *P. aeruginosa* as a Model Gram-negative Biofilm Former

Pseudomonas is a ubiquitous, aerobic Gram-negative genus (Frangipani, Slaveykova et al. 2008). It can respire oxygen at concentrations as low as 3 μ M dissolved oxygen or in the absence of oxygen can respire using nitrate as an electron acceptor (Alvarez-Ortega and Harwood 2007). It is found in moist environments such as soils, lakes, and most human-made environments as well as various host microbiomes in mammals, fish, and plants. The genus was initially identified in 1882 by Gessard and termed by Migula in 1894 as *Pseudo* (“false”) *monas* (“single-celled organism”), indicating that it is unicellular (Lister, Wolter et al. 2009). The identified specie of the genus, *aeruginosa* (meaning “of blue-green color”, the color of rust) has later emerged as one of the key model organisms for the study of biofilm formation.

P. aeruginosa is considered to be a ubiquitous nosocomial pathogen and ranks as the fifth of overall frequent hospital-acquired pathogens (Wolter and Lister 2013). It is associated with infections of airways, wounds, medical devices such as catheters and joint replacements, burns, hospital equipment (Deretic 2000) (**Figure 1.5**).

The ubiquitous *Pseudomonas aeruginosa* is also...

...*Pseudomonas aeruginosa*, the opportunistic pathogen.

Figure 1.5 *P. aeruginosa* is a versatile organism which exists in many environments, including the human body. When a patient's immune system is compromised, the opportunistic *P. aeruginosa* can be found in various related infections. Images are taken from <https://thenounproject.com> under creative commons license.

The bacterium can be part of non-sterile body sites, such as the skin, mouth and nostril flora; and would be considered benign, or commensal, merely colonizing the surface. It is regarded as an “opportunistic pathogen” and can penetrate the internal layers of that organ, through wounds, or to the inner parts of the airways and lungs for example, through initial attachment facilitating a biofilm and leading to chronic infection (Wagner, Sommer et al. 2016) (**Figure 1.6**).

Figure 1.6 Examples of chronic infections predominated by *P. aeruginosa* biofilm. Scanning electron microscope (images.fineartamerica.com), contact lens-related eye infection (www.honolulugeclinic.com), infected lung tissue of cystic fibrosis patient (microbewiki. keynon.edu), urinary tract infection (jucing-for-health.com), neonatal and pediatric-related infection (dermaamin.com), chronic wound infection (www.medetec.co.uk), folliculitis skin related infection (callisto.ggsrv.com), nail infection (www.danderm-pdv.is.kkk.dk).

1.7 Antibiotic testing strategies

Given its innate antibiotic resistance and its ability to form biofilms that further protect *P. aeruginosa* during infection, it is clear that further work needs to be done to identify effective treatments to control this important pathogen. However, most therapeutics are tested against planktonic cells and do not address the issue of biofilms. As noted above, biofilms can be up to 1000 fold more resistant to antibiotics and hence, biofilms may need to be considered as part of antibiotic testing. The following sections discuss several approaches to testing antimicrobial agents.

Planktonic bacteria

For susceptibility testing of *P. aeruginosa* to antibiotics, the standard high-throughput procedures involve broth microdilution techniques in tubes or microtiter plates. The straightforward parameter that can be measured is turbidity of the bacterial suspension. The sample which shows a drop in the optical density (O.D) correlates to a minimal inhibitory concentration (MIC) and can be estimated even with the naked eye; or more accurately, with the use of absorbance measurement using a microplate reader. When using readers the threshold that is usually reported is MIC₅₀ the concentration that leads to 50% inhibition of growth (Amyes, Miles et al. 1996). MIC allows a relatively quick and easy determination of a concentration range of inhibition, with one major drawback, experimental values depend heavily on experimental conditions (e.g. volume of culture, temperature, antibiotic stock preparations and storage) (Wiegand, Hilpert et al. 2008). Crisscrossed gradients of two antimicrobials can be prepared on a challenge plate, termed “checkerboard”. The combined

treatment can yield a cumulative MIC which is lower than each drug alone, thus exhibiting synergism. Synergy is quantified by the fractional inhibitory concentration (FIC), where the inhibitory concentration of each drug in the combination is divided by its MIC alone, summed FIC values lower than 0.5 are considered synergistic.

The clinical standards institute (CLSI), which systematizes protocols for clinical laboratories worldwide advocates on using the Mueller-Hinton Broth II (MHB) when testing for susceptibility (CLSI 2012). MHB is a rich media based on beef extract broth and casein hydrolysate in physiological pH (7.3 +/- 0.1) (Mueller and Hinton 1941). Initially developed for susceptibility testing of specific organisms, MHB was found to be satisfactory for growth of virtually all common aerobic human pathogens in a non-selective manner. MHB contains starch which allows for more efficient absorption of antibiotic intermediates and bacterial toxins, and also contains a defined concentration of Mg^{+2} and Ca^{+2} ions which stabilize antibiotics, limit adsorption to plastic, as well as simulate physiological blood concentrations (Koeth, King et al. 2000, CLSI 2012, Bakthavatchalam, Pragasam et al. 2018).

An additional parameter to quantify the efficacy of an antibiotic is the minimal bactericidal concentration (MBC), which is the concentration required to kill at least 99% of bacteria in solution (Amyes, Miles et al. 1996). When the MBC is $\leq 4x$ than MIC, the compound is considered to be bactericidal, and if the MBC is $> 4x$ the MIC, it is bacteriostatic (CLSI 2012). One limitation of the MIC and MBC assays is that they typically require 24 hours or longer to obtain complete results. To address this, alternative methods have been developed. One such

method involves the dye resazurin (Alamar Blue) a highly water-soluble molecule, which can penetrate cell membranes and is reduced by reaction with Nicotinamide-adenine-dinucleotide (NADH) (Candeias, MacFarlane et al. 1998). A relative short incubation period, low reagent price and the ease of use make this assay a suitable candidate for standard susceptibility testing (Sandberg, Schellmann et al. 2009) (**Figure 1.7**).

Figure 1.7 Microtiter plate broth microdilution method combined to determine MIC, combined with REMA assay to determine MBC. A challenge plate is prepared with a gradient of antibiotic concentrations, in which bacteria are inoculated at $\sim 5 \times 10^5$ CFU/mL and left overnight. MIC is the concentration that inhibits 50% of growth. The REMA assay measures MBC: Resazurin in wells which contain active cells becomes reduced (pink) and highly-fluorescent, otherwise remains blue. MBC is the concentration which results in 99% killing of bacteria.

The most accurate manner by which MIC/MBC parameters can be assessed involves serial dilutions of sample aliquots following turbidity measurements and enumeration of colony forming units (CFU) following treatments. Though it is considered a more laborious method, it constitutes a test which is less prone to variation assuming each CFU originates from a single bacteria (Hazan, Que et al. 2012).

Biofilm bacteria

To date, there are no internationally recognized standard procedures to define biofilm antimicrobial susceptibility profile (Malone, Goeres et al. 2017). Currently, biofilm antimicrobial susceptibility is studied using a wide range of different experimental systems, high-throughput or high-content. Two commonly used systems for high-content assessment are the Technical University of Denmark (DTU) biofilm flow cells (Tolker-Nielsen and Sternberg 2011) and the Center for Disease Control and Prevention (CDC) bioreactor (Garey, Vo et al. 2009). Both represent continuous culture systems where the fresh medium is continually supplied to support biofilm growth.

The DTU system has contributed extensively to the understanding of biofilm development usually studied with confocal laser scanning microscopy (CLSM), by continuous time course measurement or at specific intervals. For antibiotic susceptibility, a commonly used technique involves staining bacteria with the “Live/Dead” kit, whereby samples are analyzed by two stains, one that stains the live bacteria population (with intact membranes) and the other that stains dead cells (with compromised membranes). In a research from recent years, the flow cell system was utilized in tandem with isotopic labeling of amino-

acids to study dynamics of susceptibility of bacteria to colistin in different stages of the biofilm life-cycle (Chua, Yam et al. 2016). The proteomic analysis allowed for translation quantification of specific resistance related enzymes in *P. aeruginosa* under colistin stress, and the effect on other biofilm mechanisms such as virulence.

The CDC biofilm reactor is a system used primarily to examine the efficacy of antimicrobial monotherapies and combinations under conditions, which mimic the kinetics, and dynamics of drugs in the host body. For example, a bioreactor system was used to examine the efficacy of derivatives of novel antimicrobial peptides (Sánchez-Gómez, Ferrer-Espada et al. 2015); because the dynamic properties of the original drug were known, the system was utilized to study *P. aeruginosa* biofilm killing kinetics by its derivatives, thus minimizing the need for *in-vivo* experiments. This system can also be combined with CLSM and other techniques to examine biofilms at different developmental stages.

Both systems have distinct advantages such as: determining susceptibility under continuous culture conditions which better represent the host environment, and biofilm development under flow conditions, or the versatility of possible experimental setups. However, neither of these systems is amenable to high-throughput screening for a number of reasons: first, they require relatively complicated setups, involving pumps, tubing and glassware; the user has to have a certain level of expertise before meaningful data can be collected. Second, they provide a relatively low number of replicates per experiment, each flow cell contains three separate channels, requiring more

than one flow cell to be used for each experiment further complicating setup (Heydorn, Ersboll et al. 2000). The bioreactor contains 8 rods, which hold three biofilm samples each; one bioreactor can be used to study one antimicrobial regime at a time, again complicating setups.

A number of high-throughput microtiter based assays are available for evaluating the effect of compounds against biofilms. Biofilms that form in these microtiter plates are typically quantified using crystal violet (CV) as a proxy for the total amount of biofilm present (cells and EPS). CV binds to different negatively charged molecules and especially the peptidoglycan in the bacterial membranes, DNA and EPS constituents (Feoktistova, Geserick et al. 2016).

One significant modification of the microtiter plate biofilm assay is the “Calgary Biofilm Device” (CBD) (Ceri, Olson et al. 1999): a modified plate lid with pegs that hang down into the medium in the wells of the microplate. Biofilms would develop on the pegs as well as in the wells, but for these assays, only the biofilm on the pegs are quantified. A particular advantage of this system is that it quantifies active biofilm formation, where the bacteria have to attach to the pegs rather than quantifying settlement of bacteria from the liquid phase onto the bottom of the plate. Additionally, the lids and associated biofilms can be easily transferred between plates and challenged with antimicrobials. The peg biofilms can then be used to determine the minimal biofilm inhibitory concentration (MBIC). This measure is analogous to the MIC, as it is based on bacterial growth quantification (Macia, Rojo-Molinero et al. 2014). For instance, by measuring changes to samples O.D, planktonic and dispersed

cells always exist in the solution; lower turbidity can thus be a proxy to lower biofilm growth.

One of the first protocols describing MBIC testing with CBD has already clearly shown that these inhibitory values were much higher than conventional MIC values, and continued to increase as biofilms were allowed to grow for longer durations (Moskowitz, Foster et al. 2004). The same work has revealed that *P. aeruginosa* isolates from CF patients were displaying varying degrees of resistance, at most cases rendering antibiotic administration based on MIC inefficient in chronic infections.

An important parameter that can be determined is the minimal biofilm eradication concentration (MBEC), which is the concentration that results in eradication of 99% of biofilm bacteria. As for the MBC testing above, the REMA assay can be utilized to assess the MBEC (Dalecki, Crawford et al. 2016). Conversely, the synergy of two antimicrobials can be evaluated by a checkerboard antimicrobial challenge, as in FIC, by a fractional biofilm eradication concentration (FBEC) if a combination yields lower MBEC values than monotherapy (**Figure 1.8**).

Figure 1.8 The use of CBD to exclusively measure susceptibility of the biofilm fraction of bacteria. The challenge plate depicted here is organized as a checkerboard assay, with criss-cross gradients of two drugs, horizontally and vertically. MBIC is measured as turbidity, and the REMA assay is utilized to measure MBEC.

Despite the inherent advantages of the CBD, it is not suitable for CFU measurements. The suggested protocols (Ceri, Olson et al. 1999, Moskowitz, Foster et al. 2004) involve mechanical disruption of biofilms to detach bacteria and constituted in this research a high variance in preliminary experimental results. To overcome this problem, a novel method exists that utilizes biofilms grown on 5 mm glass beads (Konrat, Schwebke et al. 2016). Beads-grown biofilms show a high level of repeatability, essentially the same techniques can be applied to bead biofilms, but beads can be more readily collected and handled.

The bead biofilm system allows performing time-dependent killing assays, necessary for understanding the efficacy of antimicrobial agents (Schwalbe, Steele-Moore et al. 2007). All discussed parameters above provide snapshot assessments of susceptibility, conventionally after 24-hour exposure, simulating a once-daily antibiotic regimen. Time-kill curves, of CFU values versus exposure time, allows calculating the minimal duration of killing (MDK), which is an important parameter when determining efficacy against slow-growing bacteria, such as biofilm bacteria (Brauner, Fridman et al. 2016). MDK₉₉ is the time point by which 99% of the population is eradicated, i.e. 2 log₁₀ reduction in CFU. By the same convention, a timed series of CFU determination during the course of 24 hours provides an understanding of antimicrobial killing kinetics.

The underlying problem for prediction of treatments to efficiently eradicate biofilms, apart from lack of comparative standards, is the duration of treatments (Malone, Goeres et al. 2017). High efficacy is dependent both on

high concentrations of antibiotics and on long time scales, which in turn are dependent on diffusion through the matrix and different metabolic rates. Even when drug combinations are utilized, concentrations remain high and involve risks for toxicity in patients as well as deterioration of the quality of life. The most successful treatments still have no assurance that biofilms will not prevail somewhere, remaining a source of possible acute re-infection by dispersed cells (Bjarnsholt, Alhede et al. 2013). When evaluating new strategies to combat biofilms, the primary considerations are shortening treatment times (lower MDK₉₉) to reduce the risk of emergence of antimicrobial resistance over prolonged exposure; and reducing relevant antibiotic concentrations required to eradicate biofilms to increase treatment efficacy (faster and greater killing effect).

1.8 Research Gaps

The development of biofilm-associated infections is one of the leading mechanisms that represent a niche for AMR to spread. Biofilms represent a diffusion barrier to antimicrobials, which protect bacteria. Standardized techniques used to study susceptibility of bacteria to antimicrobials focus predominantly on planktonic bacteria (Fournier, Drancourt et al. 2013), despite the suggestion that the majority of infections, especially chronic infections, are associated with biofilms. One strategy to better treat biofilm infections would be to initiate dispersal, where the dispersed cells are more susceptible to antibiotics (Kostakioti, Hadjifrangiskou et al. 2013, Yu, Su et al. 2015, Ren, Wu et al. 2016).

The challenge of investigating the effectiveness of biofilm dispersal as part of a treatment strategy is that dispersal agents have yet been used extensively in a clinical setting (Fleming and Rumbaugh 2017). Furthermore, the use of antibiotic combinations in tandem with biofilm dispersal has never been applied clinically. The combined efficacy has the potential to allow for high synergy at *in-vivo* achievable antimicrobial concentrations, suggesting that the antimicrobial effect will occur in *in-vivo* conditions, during patients' therapy.

1.9 Aims and Scope

Using a strain of *P. aeruginosa* that can be induced to disperse as a model organism, this study aimed to provide a proof of concept of the therapeutic potential of biofilm dispersal. The study has three distinct but interrelated chapters that aimed at:

Chapter 2: Evaluating biofilm dispersal as a possible adjunct therapy. A *P. aeruginosa* strain containing an arabinose-inducible PDE enzyme was used to demonstrate that c-di-GMP mediated biofilm dispersal has the potential to increase antimicrobial synergy, by enhancing killing efficiency and lowering drug concentrations required for biofilm eradication.

Chapter 3: Assessing the risk of development of resistant variants due to exposure to antibiotic stress. Morphotype variants were compared to the parental strain for motility, biofilm production and MIC parameters. The effect of continuous induction of c-di-GMP mediated biofilm dispersal on antibiotic therapy was further evaluated.

Chapter 4: Demonstrating the potential of c-di-GMP mediated biofilm dispersal as last resort strategy for treating bacterial biofilms of AMR strains. A colistin susceptible and a resistant isogenic pair of PAO1/*p_{BAD}-yhjH* strains were generated and fully characterized in order to investigate the potential for c-di-GMP mediated biofilm dispersal to eradicate biofilms of AMR strains.

Chapter 2: Evaluation of c-di-GMP Mediated Biofilm Dispersal as a Strategy to Restore Antibiotic Efficacy

Based on the publication “*In-vitro* evaluation of biofilm dispersal as a therapeutic strategy to restore antimicrobial efficacy” (Roizman, Vidaillac et al. 2017).

2.1 Introduction

The current strategy to eradicate chronic bacterial biofilm infections involves long-term combinatorial antibiotic treatments at high dosages and the surgical removal of the infected tissue/foreign body (Høiby, Bjarnsholt et al. 2014). A notable example are treatments administered to CF patients, with chronic *P. aeruginosa* infections; combinations of broad-spectrum antibiotics (such as carbapenems) and specific anti-pseudomonal antibiotics (such as tobramycin) are administered to combat phases of acute infection and as a preventative measure against resistance development (Foweraker, Laughton et al. 2009). It is additionally known that these combinations have the potential for synergy, but experiments with clinical isolates show varied results. Prolonged antibiotic stress has been shown to eventually facilitate MDR, as exhibited in *P. aeruginosa* isolates from CF patients (Jansen, Mahrt et al. 2016).

Recently, there has been a growing interest in combining chemical agents that interfere with bacterial communication or signaling pathways with broad-spectrum antibiotics to treat biofilm-associated infections (Jakobsen, Tolker-Nielsen et al. 2017). Thus, through attenuation of biofilm formation, these

quorum sensing inhibitors restore the killing efficacy of antimicrobial treatments (Kostakioti, Hadjifrangiskou et al. 2013). C-di-GMP is a secondary messenger with multiple regulatory functions, including a central role in controlling biofilm formation. Higher intracellular levels of c-di-GMP enhance biofilm formation, while lower levels lead to biofilm dispersal with the potential to, at least partially, restore antimicrobial susceptibility. It has been recently shown that NO mediated c-di-GMP degradation leads to higher killing potential by tobramycin, of MDR clinical *P. aeruginosa* isolates from CF patients (Howlin, Cathie et al. 2017).

Even though all of the above phenomena are recognized, i.e. antibiotic combinations are preferred at treating chronic biofilm-related infections, and, c-di-GMP mediated dispersal potentiates the efficacy of specific antibiotics. There is a lack of proof-of-concept studies combining dispersal and combined antibiotic therapy. Due to the lack of clinical laboratory standards to test antibiotic synergy against biofilm models, *in-vitro* systems should first be optimized to investigate mature samples (Malone, Goeres et al. 2017). If a combinatorial treatment can lower the necessary drug concentrations and treatment durations, to efficiently eradicate biofilms - it can result in a considerable improvement of patient life quality. The present study tested the hypothesis that c-di-GMP mediated biofilm dispersal has the potential to enhance antimicrobial synergy and ultimately improve antimicrobial killing efficacy when applied to mature biofilms.

2.2 Methods

Bacterial strains: Strains of *P. aeruginosa* used here consisted of PAO1/pJN105 a vector control carrying the *araBAD* promoter inducible by arabinose, and gentamicin resistance for selection (Gm^r). PAO1/p_{BAD}-*yhjH* carrying a plasmid containing *yhjH* under the *araBAD* promoter. The YhjH PDE from *Escherichia coli* (*E. coli*) that degrades intracellular c-di-GMP. It has been transformed into the *w.t* strain of *P. aeruginosa* PAO1 and a protocol for the utilization of this system to study the effect of c-di-GMP mediated dispersal on PAO1 biofilm has been described previously (Chua, Hultqvist et al. 2015), and PAO1 *w.t*.

Chemicals: Lysogeny Broth Agar, Miller recipe (LB Agar, BD Difco) and Mueller-Hinton broth II or agar (MHB or MHA, Sigma-Aldrich) were used. Antimicrobial agents include tobramycin, imipenem, colistin, ciprofloxacin (Sigma-Aldrich), gentamycin (MP Biomedicals) and azithromycin (Tee Hai Chem).

Biofilm formation and induction of dispersal: Strains were streaked fresh from frozen stocks onto LB agar supplemented with gentamicin sulfate at 60 µg/mL. Single colonies were picked into 2 mL MHB, in 10 mL falcon tubes, strains containing Gm^r were grown with supplemented 20 µg/mL gentamicin, *w.t* without. Inoculums were grown overnight (O.N.) at 37°C with shaking at 200 rpm, following which were centrifuged 6K rpm 5 minutes. Pellets resolubilized in 6 mL MHB, and their turbidity was measured as O.D at 600 nm with a UV-Vis Spectrophotometer (Shimazu). Serial dilutions in MHB were initially used for colony forming units (CFU/mL) enumeration versus O.D₆₀₀.

Inoculums at 0.005 O.D₆₀₀ final turbidity, concurring to $\sim 10^6$ CFU/mL, as advised by CLSI (CLSI 2012, Malone, Goeres et al. 2017), for an initial inoculum size of $\sim 5 \times 10^5$ - 10^6 CFU/mL and is used in all further experiments.

Different static, *in-vitro* systems were used to grow biofilms, 24-well and 96-well microplates (NUNC™, Thermo-Fischer) were used in high-throughput with Infinite Pro 200 microplate reader (Tecan). Confocal experiments (CLSM) with biofilms were conducted using ibidi® eight-well (200 µL) glass bottom µ-slides. Where indicated the Calgary Biofilm Pin Lid Device (CBD) (NUNC™, Thermo-Fischer) (Ceri, Olson et al. 1999) 96-well peg lid system or 5 mm glass beads (Merck) were used as substratum for biofilm growth. Biofilms were grown in each system using MHB unless indicated otherwise, at a 37°C incubator, without shaking. For experiments with duration of over 1-day a washing and media replenishing step were applied daily: wells were washed with 0.9% NaCl (in the case of CBD a wash plate was used) and replenished with fresh MHB, samples were maximally grown for 3 days. If CBD was used the peg lid was transferred daily into a clean 96-well plate containing 200 µL 0.9% NaCl in each well to rinse off loosely attached cells and then transferred to a plate with fresh MHB medium.

Arabinose-induced dispersal: To induce c-di-GMP mediated dispersal and test its effects on the model PAO1/p_{BAD}-*yhjH* biofilms, samples were exposed following wash with 0.9% NaCl, to fresh MHB containing 0.25-1% weight per volume (w/v) arabinose for 4 hours (Chua, Tan et al. 2013). Control experiments were performed in parallel with PAO1/p_{BAD}-*yhjH* using an arabinose-free medium, and with the control strains. The effects of c-di-GMP

mediated dispersal were validated as a change in sample turbidity at O.D₆₀₀ and CFU/mL measurement of the resulting sample medium.

Biofilm production quantification: Crystal violet (CV) staining was performed with a stock solution of 0.01% volume per volume (vol/vol) CV (Sigma-Aldrich) in dH₂O (MilliQ) (O'Toole 2011, Chua, Hultqvist et al. 2015). 24-well plates were used to grow 1-day biofilms, liquid from O.N. biofilm growth was carefully pipetted out (not disturbing the biofilm at the bottom and on sides of wells) and washed with 0.9% NaCl. 1 mL of CV solution was added, and the plate left at room temperature with shaking at 80 rpm, for 30 minutes. The excess stain was then removed by pipetting and the plates immersed in water, decanted and left to dry upside down on the bench. The stain was then solubilized with 1 mL ethanol in each well and plates left on a shaker at 80 rpm for 15 minutes. CV Absorbance (Abs) at 590 nm was measured using an Infinite Pro 200 microplate reader (Tecan).

Inhibitory antibiotic concentrations: Minimum inhibitory concentrations (MIC) were assessed by manual un-aided inspection of sample growth followed by turbidity O.D₆₀₀ measurement in a microplate reader. Experiments with planktonic samples had 5×10^5 - 10^6 CFU/mL in each well, as advised by CLSI (CLSI 2012). MIC was determined by broth microdilution, using two-fold dilution gradients of different antimicrobials. Following 24 hour incubation at 37°C, sample turbidity was measured as O.D₆₀₀. Values higher than 3 times the blank controls wells were considered as indicative of growth, thus below the MIC range. Multiple experimental replicates were performed and included three independent biological replicates for each condition tested as well as two

technical replicates. Experimental results are expressed as the average values $\pm$ standard deviation (STDEV) with N=6.

Minimal bactericidal concentration (MBC): To determine the MBC values directly over-and-above of MIC values, two techniques were employed. First, 10 μ L aliquots were sampled from each well in each concentration above the MIC. Samples were drop plated on LB agar and incubated O.N. at 37°C, and at the following day (after 20-24 hours) visually inspected for growth. MBC was determined as the antibiotic concentration in wells that did not contain any viable bacteria as could be detected.

Second, MBC was evaluated by the resazurin microtiter assay (REMA) (Sandberg, Schellmann et al. 2009). 10 μ L of resazurin stock solution, prepared as 0.3 mg/mL (~1.2 mM) were added to each well to a final concentration of 15 μ g/mL (60 μ M). Plates were incubated for 3-4 hours at 37°C, and fluorescence was measured in an Infinite Pro 200 microplate reader (Tecan) with excitation at 550 nm and emission at 590 nm under automatic optimal gain. Wells' fluorescence values equal or higher than 3 times the fluorescence value of the control blank wells were considered viable, and thus, under the MBC range.

Antimicrobial synergistic assays against biofilms: The synergistic potential of tobramycin and imipenem, as well as the impact of induced biofilm dispersal, were assessed in a checkerboard manner (Jenkins and Schuetz 2012). "Challenge plates" consisting of two-fold gradients of both tobramycin (0.25 to 64 μ g/mL) and imipenem (1 to 2048 μ g/mL) in MHB were prepared. Plates were prepared so that drug A was diluted in rows (horizontally) and drug B

diluted in the columns, with column 12 reserved for controls (growth and negative), column 1 and row A were monotherapy (Dundar and Otkun 2010).

Monotherapy minimal biofilm eradication concentrations (MBEC) values and fractional biofilm eradication concentration (FBEC) indices for combinations were determined for 2-3 day grown CBD biofilms in MHB. Biofilms were formed by inoculating *P. aeruginosa* into the wells of a 96-well plate at a final density of $5 \times 10^5 - 1 \times 10^6$ CFU/mL and incubation at 37°C. CBD biofilms were grown as described above for 3 days, following which induced dispersal was applied for 4 hours as described, or not. Biofilms were then transferred to the Challenge plate and incubated for 24 hours at 37°C. Following challenge, biofilms were washed in plates with 0.9% NaCl and subsequently transferred to a plate with 200 µL MHB media with 60 µM resazurin for 4 hours at 37°C, to determine the viability of the biofilm bacteria. Viability was measured by fluorescence quantification in a microplate reader, as described above for MBC. For monotherapy, MBEC values were defined as the lowest drug concentrations resulting in fluorescence values that were no more than twice the negative controls. FBEC and Σ FBEC values were calculated as previously described (Shafiei, Abdi Ali et al. 2014): A value of 1 was given to the MBEC of mono-treatment, and following the two-fold dilution manner, a value of 0.5^N (N being the number of dilutions) was given to the corresponding wells in the combined treatment. Values for both drugs were summed to Σ FBEC, combinations with a Σ FBEC value ≤ 0.5 were considered synergistic.

Biofilm time-kill kinetics to assess MDK₉₉: Antibiotic susceptibility was determined as a factor of time. Here, biofilms were grown for 3 days using

5 mm glass beads (Konrat, Schwebke et al. 2016) in MHB. Two beads (representing two technical replicates, verifying reproducibility) were placed into each well of a 24-well microtiter plate and exposed to fresh MHB containing 1% (w/v) arabinose or arabinose free medium (control samples). After 4 hours, samples were treated with imipenem and tobramycin (8 µg/mL) alone or in combination. Biofilms were removed from the beads at different time points by alternating 10-second sonication in a water bath (at 37 kHz) and 10 seconds of vortexing (both at 100% power). Bacterial suspensions were subsequently serially diluted and drop-plated onto LB agar plates. After 24 hours incubation at 37°C, the colony forming units were determined as average of CFU/bead and plotted against time. Experiments were performed in five independent biological replicates to ensure reproducibility; results are expressed as the average value +/- STDEV, (N=5).

Confocal Microscopy: The efficacy of the combined antibiotic therapy was also examined using confocal laser scanning microscopy (CLSM, Zeiss 780, Germany). Biofilm samples were grown on eight-well (200 µL) glass bottom µ-slides for 3 days before exposure to imipenem and tobramycin (at 8 µg/mL) alone or in combination. After the 24 hours antibiotic challenge, samples were stained for 10 minutes, using the LIVE (Syto-9)/DEAD (Propidium Iodide, PI) BacLight™ Bacterial Viability Kit®. Syto-9 and PI fluorescence measurements were conducted by excitation with 488 and 561 nm laser lines, respectively. Four images (considered as technical replicates) were acquired in Z-stacks for each sample. The ratio of average bio-volumes ($\mu\text{m}^3/\mu\text{m}^2$) calculated for live and dead stained populations in each image was quantified using COMSTAT 2 ImageJ plugin (Heydorn, Nielsen et al. 2000). A constant threshold of 50

was set for each channel. Experiments were performed in five independent biological replicates to ensure reproducibility; results are expressed as the average $\pm$ STDEV (N=5).

Statistical analysis: Statistical significance analysis (using multiple student's t-test or two-way ANOVA, where indicated) using software recommended method for each experiment and final graph plotting were all conducted using Prism GraphPad 7.

2.3 Results

Antimicrobial susceptibility testing against planktonic cultures

PAO1 strains Inocula at O.D₆₀₀ 0.01 were preliminarily found to correlate to 1.5×10^6 CFU/mL (**Figure 2.1C**) yielding a final inoculum density of 5×10^5 - 10^6 CFU/mL in experimental wells for all experiments, as instructed by CLSI (CLSI 2012). To determine the optimal medium for subsequent c-di-GMP mediated dispersal assays, biofilms were formed for 24 hours in four different media, ABTGC (Chua, Hultqvist et al. 2015), MHB, TSB (Sigma-Aldrich) and LB and the dispersal response of these biofilms was determined. Dispersal was induced by adding arabinose, which controls the expression of the phosphodiesterase *yhjH* and dispersal in the presence and absence of arabinose after 4 hours of incubation was also compared.

The amount of biofilm formed, as determined by CV staining, was similar for ABTGC, TSB and LB (Abs₅₉₀ between 0.125 and 0.15). In contrast, there was significantly more biofilm formed in MHB (0.21 $\pm$ 0.002) compared to the

other media (**Figure 2.1A**). While arabinose was able to induce dispersal of biofilms grown in all media, dispersal was only significant for MHB grown biofilms, where approximately 55% of the biofilm was dispersed after arabinose addition (Abs_{590} 0.11 $\pm$ 0.001). It is unknown why dispersal had a stronger effect in MHB media or why such difference occurs in CV stain as compared to other media. It was suggested in other works that the higher Ca^{+2} concentrations might be the main effector for PAO1 (Jain, Parida et al. 2013). In general, all other media did not show any significant difference in CV stain with or without arabinose addition (Average values between 0.09-0.14). Dispersal was also quantified by measuring the O.D₆₀₀ of the planktonic cells (**Figure 2.1B**), which increased 47% to 66% for the different media used, relative to the control without arabinose induction. Similarly, the CFU counts were higher in the planktonic phase after arabinose addition, where the CFUs increased 140% ($\pm$ 3.35%) for ABTGC-grown cells and 232% ($\pm$ 0.28%) for MHB biofilms. MHB showed the most significant difference between treated and untreated samples (t-test, p-value < 0.001). Based on these results, subsequent experiments used MHB medium since this medium showed the highest amount of total biofilm formed (Abs_{590} of 0.22 $\pm$ 0.003) and the highest amount of dispersal (55%). Further, MHB is the recommended medium for MIC testing based on the standardized protocol (CLSI 2012).

Figure 2.1 The effect of medium on the c-di-GMP mediated dispersal response of *Pseudomonas aeruginosa* PAO1/*p*_{BAD}-*yhjH* biofilms, carrying the arabinose-inducible phosphodiesterase *yhjH*, were formed in ABTGC, MHB, TSB and LB for quantification of biofilm formation and dispersal. The amount of biofilm formed (A) and dispersed upon arabinose addition as measured by CV staining (Abs₅₉₀), resulting changes to sample turbidity (O.D₆₀₀) (B) following induced dispersal. Establishment of initial inocula conditions for all further experiments (C) as turbidity vs. CFU/mL and a comparison of induced dispersal as CFU/mL (D) of the supernatant above the biofilms for treated and untreated samples in ABTGC and MHB media. Values represent the average of 3 independent experiments, +/- STDEV; **: p-value < 0.001 multiple t-test, using the Bonferroni-Dunn method, $\alpha=0.05$.

To ensure that the plasmid vectors used did not affect the drug susceptibility profile, imipenem MICs and MBCs values of *P. aeruginosa* PAO1 were compared to *P. aeruginosa* /*p*_{BAD}-*yhjH* and *P. aeruginosa* / pJN105 (Figure 2.2

A and **B**). On the basis of the O.D₆₀₀ measurements, no difference in MIC values was observed (**Figure 2.2A**). All three strains were completely inhibited at 1 µg/mL and showed approximately a 50% growth reduction at 0.5 µg/mL. In contrast, there was no significant difference in the MBC for the three strains as measured using the REMA assay, which monitors respiratory activity (**Figure 2.2B**). The MBC of imipenem for these strains was 1-2 µg/mL.

Subsequently, the MIC and MBC values were determined for imipenem and colistin (**Figure 2.2 B and C**) as well as tobramycin and azithromycin (**Figure 2.2 D and E**) specifically against *P. aeruginosa* /p_{BAD}-yjhH. Both imipenem and colistin showed identical MIC and MBC patterns, where 1 µg/mL of both compounds completely inhibited growth or activity of *P. aeruginosa*. Tobramycin was inhibitory between 0.5-1 µg/mL while azithromycin showed a MIC value of 4-8 µg/mL. Tobramycin and azithromycin differed in MBCs, where 4 µg/mL of tobramycin was bactericidal compared to 8 µg/mL for azithromycin. Thus, the difference in activity between these two antibiotics was more apparent in these assays, where tobramycin showed a 4-fold increase between MIC and MBC but azithromycin showed a maximum 1-fold increase in those values. The values observed here, for both the MICs and MBCs are consistent with published results (**Table 2.1**).

Figure 2.2 MICs (O.D.₆₀₀) and MBCs as measured using the REMA assay (emission at 590 nm) for four antibiotics. MICs (**A**, **C** and **E**) and MBCs (**B**, **D** and **F**) were determined for imipenem against the *w.t.* *P. aeruginosa* PAO1 strain, PAO1/*p_{BAD-yhjH}* and PAO1/pJN105 carrying the empty vector (**A** and **B**), while the MIC and MBCs were determined for imipenem & colistin (**C** and **D**) or tobramycin & azithromycin (**E** and **F**) for PAO1/*p_{BAD-yhjH}*. All measurements were made following 24-hour exposure at 37°C. All experiments were performed in triplicate, and the error bars are +/- STDEV. Antibiotic concentrations are shown on a log₂ scale (thus the control with 0 $\mu\text{g/mL}$ concentration could not be plotted).

Because the REMA method is not a commonly used standard for defining MBCs, the results obtained by this method were subsequently compared with MBC results by the drop-plate method (**Table 2.1**). Visual inspection and O.D₆₀₀ counts gave comparable MICs for all four antibiotics. These were similar to published values or were different by one dilution (for example, 0.5-1 µg/mL compared to 1-2 µg/mL for imipenem). The MBC as determined by drop plating at most 2-fold higher than the value obtained using the REMA assay for all antibiotics tested. REMA was found to be suitable for consecutive high-throughput microplate-based assays.

Table 2.1 Evaluation of MIC and MBC of 4 different antibiotics for PAO1/*p_{BAD}-yhjH* by different assays and comparison to literature results.

Antibiotic	MIC			MBC			References
	Visual reading [µg/mL]	Reader OD ₆₀₀ [µg/mL]	Literature [µg/mL]	Drop plate [µg/mL]	REMA Emm ₆₀₀ [µg/mL]	Literature [µg/mL]	
Imipenem	0.5-1	0.5-1	1-2	2-4	0.5-1	2-4	Riera et al (2011) Meletis et al. (2012)
Colistin	0.5-1	0.5-1	1-2	2-4	0.5-1	2-4	Hermann, et al. (2010) Hengzhuang et al. (2012)
Tobramycin	1-2	2-4	2-4	2-4	2-4	2-4	Mah et al. (2003) Tré-Hardy et al. (2010)
Azythromycin	16-32	16-32	16-32	32-64	16-32	32-64	Buyck et al. (2012) Nalca et al. (2006)

Validation of the time of biofilm formation and the amount of arabinose to be used for induction of c-di-GMP mediated dispersal

Experiments were performed to verify arabinose mediated induction of c-di-GMP mediated dispersal by the PAO1/*p_{BAD}-yhjH* strain and that arabinose addition did not alter the dispersal response or biofilm development in the absence of the YhjH phosphodiesterase. For PAO1, 24-hour grown biofilms

exhibited lower MBEC values than 48 and 72 hours (2-3-day grown biofilms) (Wu, Moser et al. 2015). It has also been shown that MBEC values increase by up to 1000 fold for 3-day biofilms (“mature”) compared to earlier time points (Hengzhuang, Wu et al. 2011). Therefore, to determine if the time of biofilm development affected the antibiotic resistance profiles in the systems used here, biofilms were grown for 24, 48 and 72 hours and compared for antibiotic responses. Similarly, the concentration of arabinose used in the literature to induce the *araC* promoter varies from 0.25% and 1%, and therefore, a range of arabinose concentrations was tested here to optimize induction of dispersal (Chua, Tan et al. 2013).

Biofilms of the three strains PAO1/*p_{BAD}-yhjH*, PAO1/*pJN105* and PAO1 *w.t* were grown in 24-well microplates for periods of 24, 48 and 72 hours (1-3 days) with daily media replenishment as described. Samples were dispersed by arabinose addition and 4-hour incubation. Significant differences were evident by CV stain between *p_{BAD}-yhjH* and the two control strains (Abs₅₉₀ of 0.11 versus 0.14) only for biofilms grown for 72 hours after induction with 1% arabinose ($p < 0.001$), (**Figure 2.3A**). Generally the biofilms of all 3 strains became less varied after 48 and 72-hour growth and might indicate a change in the bacterial population in the samples, resulting in similar EPS production. Turbidity measurements of samples supernatant O.D₆₀₀ showed no significant differences following dispersal with arabinose at any concentration. Arabinose exposure seemed to results in some level of dispersal from biofilms not related to any specific strain and might hint the involvement of a different mechanism.

Figure 2.3 Effects of 4-hour arabinose (Ara) induction on 24, 48 and 72-hour grown biofilms of PAO1 strains *p_{BAD}-yhjH*, *pJN105*, and *w.t* grown in 24-well plates. **(A)** Remaining biofilm stained by crystal violet (CV) measurement (Abs₅₉₀), **(B)** Turbidity O.D₆₀₀ and **(C)** CFU/mL of dispersed cells in the sample supernatant. Results were analyzed by two-way ANOVA. Statistically significant differences are given as corresponding p-value, interpretation: ** <0.01 *** <0.001 **** <0.0001. Experiments were performed in triplicates (N=3) and plotted as average values +/- STDEV.

CFU/mL measurements of samples supernatant indicate a significant trend following the 4 hours of arabinose-mediated dispersal (**Figure 2.3C**). For biofilms grown in this system, the growth stage that seems to exhibit the most

profound differences between dispersed samples to controls are biofilms grown for 48 hours and more. For $p_{BAD^-}yhjH$ arabinose induced 4-hour dispersal resulted in an increase in CFU/mL of samples supernatant, from 1.8×10^8 in the control to 5×10^8 and 5.65×10^8 for 0.25% and 1% arabinose respectively more than 2.5 - 3 times accordingly. 1% arabinose was more significant at this stage as indicated by two-way ANOVA ($p < 0.001$) in relation to the controls pJN105 and PAO1 *w.t* under the same conditions (1.4×10^8 and 0.7×10^8 respectively).

Biofilms of all strains grown for 72 hours prior to induced c-di-GMP mediated dispersal follow, in principle, the same trends indicated above, but the same treatment is more significant. CFU/mL of the supernatant above the biofilm was 1.13×10^8 in the control to 4.20×10^8 and 5.23×10^8 respectively for 0.25% and 1% arabinose. Both were also more significant against both control strains, most with 1% arabinose against pJN105 and PAO1 *w.t* (1.33×10^8 and 1.58×10^8 respectively). The experimental results thus indicate, that induced c-di-GMP mediated dispersal with 1% arabinose for PAO1/ $p_{BAD^-}yhjH$ grown biofilms, resulted in 3 times higher CFU/mL in the supernatant versus controls, presumably the dispersed cell fraction; results were more profound for 3-day grown biofilms.

Antimicrobial susceptibility of biofilms

3-day grown *P. aeruginosa* have been found to represent mature biofilms exhibiting 100-1000 fold higher antibiotic resistance (Herrmann, Yang et al. 2010, Hengzhuang, Wu et al. 2012). Preliminary experiments testing synergy between antimicrobial agents using REMA and CBD biofilms to establish

MBEC where were conducted in a checkerboard manner, as described in the materials and methods section. The technique allowed determining the range of effective concentrations for monotherapy and a combinatorial gradient of both drugs, the MBEC and Σ FBEC allowing comparison of fractional contribution of both drugs to synergy. Several combinations of antimicrobials were tested against 3-day grown biofilms of PAO1/p_{BAD}-*yhjH* and PAO1/pJN105. The combination depicting the highest synergy in preliminary experiments (data not shown): tobramycin and imipenem - has been chosen to be the focus of all following proof-of-concept experiments.

Arabinose induction of c-di-GMP mediated biofilm dispersal had no impact on imipenem and tobramycin mono-treatment MBEC values (2048 and 64 μ g/mL for imipenem and tobramycin, respectively) for both PAO1/pJN105 and PAO1/p_{BAD}-*yhjH* using this essay. Because tobramycin is the more efficient monotherapy, in this case, any change to its FBEC value reduces the combined Σ FBEC value more prominently. Induction of c-di-GMP mediated dispersal significantly (One way ANOVA analysis, $p > 0.1$) enhanced killing of PAO1/p_{BAD}-*yhjH* upon dual treatment with imipenem and tobramycin compared to the same combination without arabinose induction of dispersal (**Figure 2.4A**). Σ FBEC values were ranging from 0.012 to 0.017 versus 0.024 to 0.033 for non-dispersed biofilms. This effect was not observed in biofilms formed by the control strain PAO1/pJN105 (**Figure 2.4B**).

Figure 2.4 Isobolograms illustrating the relative fractional biofilm eradication concentration (FBEC) values for imipenem (IMI) and tobramycin (TOB). Absolute concentration values [μg/mL] are indicated at points. Results are the lowest $\sum$ FBEC values from five independent experiments. Combined antibiotic 24-hour treatment against 3-day grown biofilms of strains PAO1/pBAD-yhjH (**A**) and PAO1/pJN105 (**B**) with (Ara+) or without (Ara-) 4 hours induced dispersal with 1% arabinose.

Imipenem and tobramycin MBEC values in combinations were thus significantly lowered as a combination (up to 7 and 3-fold $\log_2$ decrease, respectively), after c-di-GMP mediated dispersal, compared to non-arabinose treated PAO1/pBAD-yhjH biofilms. Because this combination already exhibited a high synergy, i.e. $\sum$ FBEC values a magnitude lower than 0.5, the area under the curve (AUC) was calculated to better illustrate each isobologram (Pena-Miller, Laehnemann et al. 2013). AUC for combined treatments against pBAD-yhjH was calculated to be 7.8×10^{-5} [AU] with induced dispersal versus 3.3×10^{-4} [AU] in the absence of dispersal, for the pJN105 no difference was evident.

The stronger synergistic effect following induced biofilm dispersal was further confirmed using time-kill assessments (**Figure 2.5 A&B**). Biofilms of pBAD-yhjH

and pJN105 that were formed for 3 days on 5 mm glass beads in 24-well plates were used for this experiment. Beads were collected, and CFU/bead were determined for mono and combined imipenem and tobramycin treatments at the indicated time points. Both antibiotics were in the concentration of 8 µg/mL, 2 times higher than the original MBC, 4x MIC and 1/8 of tobramycin's MBEC, concentrations not facilitating complete biofilm eradication, but allowing analysis of synergy. The time -4 hours indicates the addition of 1% arabinose, following 4 hours induction, samples were washed and treated as indicated. Plates were sampled at -4, 0, 4, 8, 16 and 24-hour time points. Synergy was defined as 2 log₁₀ reduction in CFU/Bead in the combined treatment versus the most potent antibiotic monotherapy, tobramycin alone.

The highest reduction in CFU/Bead was at the 24-hour time point, (5.8 log₁₀ (CFU/Bead) +/- 0.34) for p_{BAD}⁻*yhjH* following induced dispersal and combined antibiotic treatment. This value did not represent an obvious synergy between both drugs, versus tobramycin mono-treatment without induced dispersal at that time point (7.36 log₁₀ (CFU/Bead) +/- 0.31), a difference of ~1.56log₁₀. Still, this combination represents 99% killing, versus all control groups, (7.85-7.77 log₁₀ (CFU/Bead) +/- 0.35), and MDK₉₉ is in the range of 16-24 hours. Lastly, lower CFU/Bead due to combined treatment could be evident after 8 hours exposure in all samples, independent of dispersal induction, but only the dispersed p_{BAD}⁻*yhjH* did not recover to 7-8 log₁₀(CFU/Bead) at the final time point, exhibiting sustained eradication.

Figure 2.5 Biofilm c-di-GMP mediated dispersal induced by arabinose enhances the synergistic antimicrobial effect of tobramycin (TOB) combined with imipenem (IMI) against *P. aeruginosa* PAO1/p_{BAD}-yjhH. Residual bacterial load (CFU/beads) after exposing a 3-day grown biofilms of *P. aeruginosa* PAO1/p_{BAD}-yjhH (**A**) or PAO1/pJN105 (**B**) treated (Ara+) or untreated (Ara-) with arabinose 1% to IMI and TOB alone or in combination with 8 µg/mL. The dashed line represents 2 log₁₀ reduction in CFU/Bead relative to untreated controls, i.e. eradication of 99% of bacteria. Results are expressed as the average value of five independent experiments +/- STDEV, (N=5).

The observation that Induced c-di-GMP mediated dispersal facilitates eradication of mature 3-day grown biofilms by the antibiotic combination was further confirmed by confocal microscopy (CLSM). Biofilms were grown using 8-well micro-slides; dispersal was induced for 4 hours with 1% arabinose and was followed by the 24-hour antibiotic challenge (as monotherapy or combination of tobramycin and imipenem at 8 µg/mL). Quantification of biofilm killing effect was conducted using syto9/PI stains, and the ratio of dead/live bacteria was calculated for each sample (**Figure 2.6**).

Following the imipenem-tobramycin combination exposure, a significant increase ($p < 0.05$) in the dead/live cell ratios (0.77 ± 0.12) was observed for PAO1/*p_{BAD}-yhjH* treated with arabinose (dispersed, Ara+), compared to the non-arabinose treated (non-dispersed, Ara-) biofilms (0.56 ± 0.13). Image analysis revealed that dispersed *p_{BAD}-yhjH* biofilms were also more susceptible to mono-treatment by tobramycin (0.69 ± 0.05) as compared to the non-dispersed control (0.52 ± 0.08 , $p < 0.001$) in this system. The most significant result ($p < 0.0001$) from comparing dead/live ratios is the killing effect seen in the combined antibiotic treatment following arabinose versus the control (0.4 ± 0.1) (**Figure 2.6B**). No significant differences in dead/live cell ratios were evident between treatments using the pJN105 strain.

Figure 2.6 Confocal Microscopy (CLSM) images (20x objective) (**A**) of 3-day biofilms of *P. aeruginosa* strains PAO1/p_{BAD}-yhjH and PAO1/pJN105 treated with arabinose 1% (Ara+) or untreated (Ara-) for 4 hours followed by 24 hours exposure to imipenem

(IMI), tobramycin (TOB) or combination of IMI + TOB at 8 µg/mL each. Biofilms were stained with Syto9 and propidium iodide prior imaging. Dead and live cells appear red and green, respectively; the scale bar is 20 µm. Live-dead ratios for biofilms after 24 hour exposure to antibiotics are given (**B**); Images from five independent experiments (N=5) were analyzed to determine dead/live ratios for each condition. Results were analyzed using two-way ANOVA (p-value interpretation: ***: p <0.0001, **: p <0.001, *: p <0.05) and plotted as average values +/- STDEV.

2.4 Discussion

Using a *P. aeruginosa* construct PAO1/p_{BAD}-*yhjH*, it was demonstrated that c-di-GMP mediated biofilm dispersal induced by arabinose enhanced the killing efficacy of a combined antibiotic treatment (**Figure 2.7** below). The findings are in agreement with published results for the combination of carbapenem agents with tobramycin (Pedersen, Pressler et al. 1987, Ciofu, Jensen et al. 1996, Hill, Rose et al. 2005, Dundar and Otkun 2010, Louie, Liu et al. 2013). It is known that the MIC's for both drugs are 0.5-1 µg/mL for susceptible strains *P. aeruginosa* it has been demonstrated *in-vitro* as well as in clinical isolates from CF patients that *P. aeruginosa*, exhibits high tolerance and eventually resistance is developed to monotherapies of both. The combination of carbapenems and tobramycin shows synergy but is effective at eradication of 99% of bacteria in high doses. The results presented here suggest that inducing biofilm dispersal via c-di-GMP depletion has the potential for a more potent antimicrobial efficacy by the use of lower drug concentrations.

Figure 2.7 Model of proven hypothesis: Inducing dispersal through c-di-GMP depletion enhances the efficacy of combined antibiotic treatment (in red and blue) against mature biofilms. Red bacteria represent dead cells, and green bacteria represent remaining viable cells. Following induced dispersal by overexpression of *yhjH* PDE enhanced killing was evident, and the ratio of dead/live bacteria in biofilms also increased.

Experiments with planktonic cultures of PAO1 strains exhibited MBC of both antibiotics to be 2-4 µg/mL. The relevant antibiotic concentrations in relation to biofilms of increased exponentially as biofilms were allowed to grow longer: 3-day grown biofilms used here, showed a significant increase in the concentrations of imipenem (of up to 1000-fold) required to eradicate efficiently (MBEC 2048 µg/mL), and 5 two-fold increase for tobramycin (MBEC 64 µg/mL). These observations are in agreement with literature (Hengzhuang, Wu et al. 2011, Kapoor and Murphy 2018), that a 3-day *P. aeruginosa* biofilm

model, can be considered “mature”, usually exhibiting MBEC of 100 - 1000 fold higher than MBC.

It is not uncommon for tobramycin to be administered once or three times daily (Bloomfield, Staatz et al. 2016), effective tobramycin plasma concentrations are usually not higher than 10 µg/mL (Burkhardt, Lehmann et al. 2006). For imipenem, it can reach $C_{\max}$ levels of ~64 µg/mL, but blood plasma levels decline below 1 µg/mL after 6 hours (Lee, Kinzig-Schippers et al. 2010). In the work presented in this chapter, a concentration equal to twice the MBC, 8 µg/mL for both antibiotics was sufficient to eradicate 99% of the biofilm population in time-kill experiments, following induced c-di-GMP mediated dispersal. These antibiotic concentrations are thus in a relevant physiological range. The synergy of the combined treatment was not prominent in the sense that a 2 log₁₀ decrease in CFU versus tobramycin monotherapy was evident. The improvement to treatment due to c-di-GMP mediated biofilm dispersal was in accomplishing MDK₉₉ of under 24-hours. Biofilms in undispersed samples and control samples were able to recover to above 10⁷ CFU/mL (7 log₁₀ CFU/Bead) at the 24-hour time point.

The experiments presented here used a genetically engineered strain to demonstrate the proof of concept that induction of c-di-GMP mediated biofilm dispersal can sensitize biofilm cells to antibiotics and that sensitization is more pronounced for combination treatments. Therefore, the results presented here suggest that compounds that induce dispersal in non-engineered or *w.t* strain can be used as adjuvants to increase the efficacy of antibiotic combinations to treat infections. For example, nitric oxide (NO) donors (Barraud, J. Kelso et al.

2015, Ren, Wu et al. 2016), QS or enzyme inhibitors (Rogers, Huigens et al. 2010, Kalan and Wright 2011), enzyme facilitated dispersal (Yu, Su et al. 2015, Gonzalez Moreno, Trampuz et al. 2017) and other techniques were shown to disperse biofilms.

NO, in particular, functions through modulation of c-di-GMP concentration by higher PDE activity (Barraud, Schleheck et al. 2009), the same dispersal mechanism investigated here. One PDE is known to be activated by NO, NbdA; other PDEs are activated by the chemotaxis sensor BdlA (Li, Petrova et al. 2014). NO is thus, related to metabolism, motility and dispersal, but also increase in virulence. NO has been shown to increase the efficacy of killing by tobramycin in clinical isolates from CF patients (Howlin, Cathie et al. 2017). When NO was used alone (as low as 500 nM active compound) no more than 10% of the biofilm is dispersed, but when combined with antibiotic treatment, it facilitated possible synergy between tobramycin and other antibiotics. Findings from this chapter provide additional evidence that dispersal through c-di-GMP degradation increases the efficacy of tobramycin, and synergy between antibiotic combinations with tobramycin. The system used here requires a considerable time (4 hours) to induce dispersal, at high arabinose concentrations (0.25-1% w/v, 16.6-66.6 mM); it is an innate problem with the araBAD expression system (Khlebnikov, Risa et al. 2000). Nonetheless, the effect seen by overexpression of the PDE YhjH was independent of the native PDE enzymes of *P. aeruginosa*. Regardless of which specific PDE involving mechanism is expressed, in response to NO exposure or by the construct presented here, inducing just one system facilitates partial dispersal which has the potential to increase antibiotic efficacy.

CV staining has been suggested in a number of recent publications to be used as a standard proxy for biofilm susceptibility assays (Haney, Trimble et al. 2018, Kapoor and Murphy 2018). It is known that CV stains both live and dead bacterial populations, and all EPS components in varying degrees. These papers and others state that CV results show a high degree of variability, and are not-directly related to viability. CV staining results in this chapter could not be used to detect significant changes to the biofilm fraction of samples, not alone used for susceptibility.

The REMA assay seems to be an efficient way for a relatively rapid (4 hours incubation and measurement) to assess susceptibility, resistance and tolerance, with a useful detection range of approximately 10^9 - 10^6 CFU/mL (Van den Driessche, Rigole et al. 2014, Kim and Jang 2017). When applied with the standard inoculum size of 10^5 - 10^6 CFU/mL it is useful for MIC & MBC measurement and high-throughput preliminary screening of MBC and MBEC values (Sandberg, Schellmann et al. 2009). Due to the detection limit, REMA is not suitable for assessment of persistence, estimated to be a 0.01% of the population (Brauner, Fridman et al. 2016). Routine measurements with REMA depend on comparison to negative and positive controls and depends on the user's threshold limit (Bueno 2014); in this research and others, 3 times the value of blank sample fluorescence was determined to indicate eradication (of down to 99.9%). Kinetic experiments have been suggested to provide useful input, and more precise measurements down to a threshold of 10^3 CFU/mL, by producing and comparing results to specie-dependent activity curve of resazurin reduction (Van den Driessche, Rigole et al. 2014, Berditsch, Jäger et al. 2015).

The CBD peg lid system is an efficient method for high-throughput exposure of virtually identical biofilm samples CFU/Peg approximately 10^7 - 10^8 (Ceri, Olson et al. 1999). Here it was shown that the CBD could be utilized in tandem with REMA to identify promising antibiotic combinations. CBD was found here not to be suitable for routine high-throughput CFU/mL count. The bead biofilm technique employed here has high reproducibility that can be used to measure parameters such as CFU/mL in multiple conditions in relatively high throughput (Konrat, Schwebke et al. 2016). It is suggested that as long as beads remain submerged in media, it is possible to use as many beads as necessary as technical replicates (Sternberg, Bjarnsholt et al. 2014). All in all, these systems show the power and versatility of static *in-vitro* systems as powerful tools for fractionation of bacteria in different phases, planktonic, biofilm and dispersed and consequently high-throughput susceptibility testing.

Although the standardization of *in-vitro* experimentation and parameters for clinical biofilm related infections is in its early stage (Høiby, Bjarnsholt et al. 2015, Malone, Goeres et al. 2017, Haney, Trimble et al. 2018, Kapoor and Murphy 2018). Consistent with the literature, it is shown here that standard techniques as described in the CLSI (CLSI 2012) can be implemented and modified to be used as general conditions for biofilm focused experiments; some notable examples: CLSI suggests strict use of MHB as a default media, $\sim 5 \times 10^5$ CFU/mL inoculum, and use of methods such as broth microdilution, to allow comparison of parameters such as MIC/MBC across species (Reller, Weinstein et al. 2009), and for use of FIC indices to identify synergy of combinatorial treatment. All of the above were adopted here while developing the methodology for biofilm models. Σ FIC estimation by broth microdilution

checkerboard assay based on fluorescence such as REMA are usually performed with a minimum of five independent replicates (N=5) (Rand, Houck et al. 1993). By extension, the Σ FBEC of imipenem and tobramycin was calculated here in a checkerboard manner using the Calgary device, following the methodologies used for planktonic cells (e.g. Σ FIC).

Σ FIC is a parameter used to identify the best synergistic drug combination in regards to MIC, i.e. inhibiting planktonic bacteria. The MIC₉₀ values of imipenem and tobramycin were 1 and 2 µg/ml, respectively. The MBEC values of Imipenem and tobramycin as mono-therapy against 3-day mature biofilms were 2048 and 64 µg/mL, respectively. The minimal concentration required for both drugs to observe a synergistic effect (Σ FBEC ≤ 0.25) against both strains was 8-16 µg/mL, regardless of dispersal. This, in turn, corresponds by convention to FBEC of 0.004 - 0.008 for imipenem, and 0.125 - 0.25 for tobramycin. Σ FBEC value of both is already ≤ 0.25 ("highly synergistic") and becomes infinitesimally small and insignificant for a drop of a 1000-fold in concentration. It is not defined what differences in the parameter indicate below "high synergism". Additionally, results show here that dispersal and combined antibiotic treatment have higher efficacy; conventionally broth microdilution experiments have to agree at least 80% of the time (Rand, Houck et al. 1993, CLSI 2012). Because, the Σ FBEC parameter is not defined below 0.25, further experiments were warranted to shed more light on the mechanism by which dispersal improves antimicrobial efficacy.

CLSM observations indicate that following c-di-GMP mediated dispersal tobramycin mono-treatment at 8 µg/mL shows significantly increased dead/live

ratio (0.7 +/- 0.05), as compared to the dispersed control (0.4 +/- 0.1, $p < 0.001$). This result suggests that tobramycin mono-treatment is sufficient to initiate killing with high efficacy. Killing is further enhanced ~10% using the combination with imipenem but only for dispersed samples (dead/live ratio = 0.77 +/- 0.12, $p < 0.0001$). No significant changes in killing between all three control groups were identified at 8 $\mu\text{g/mL}$ for both antibiotics, alone and in combination. There were also no differences in average maximal biofilm thickness, regardless of dispersal or species: control for Ara+ or Ara- for either $p_{BAD}/yhjH$ or pJN105 averaged at 20 +/- 1.67 μm , and 9.95 +/- 0.40 μm following combined treatments. It can be assumed then, that all antibiotic treatments resulted in approximately 50% reduction in biomass, out of which, only the combined antibiotic and dispersal treatment had the potential to achieve >75% killing effect.

Time/kill experiments support that observation, monotherapy with either antibiotic for 8 hours following c-di-GMP mediated dispersal, already shows 90% killing (1.1×10^7 versus 1.2×10^8 CFU/Bead), (MDK_{90}); at that time point, the combined antibiotic treatment following dispersal shows 95% killing (5.01×10^6 CFU/mL). MDK_{99} then occurs only between 16-24 hours with (6.4×10^5 versus 7.1×10^7 CFU/mL in control). A pharmacokinetic/pharmacodynamics experiment, using mice infection models or the CDC bioreactor (Garey, Vo et al. 2009) is now warranted, to determine whether the recommended dosing therapy regimens of this antibiotic combination in tandem with dispersal induction via this mechanism.

In conclusion, the data presented in this chapter serves as a proof-of-concept for future development of dispersal agents to be used along with antimicrobial combinations. Inducing c-di-GMP mediated dispersal before antibiotic treatment allows for higher efficacy potential of antibiotic combinations at the start of a regimen. However, even a combined antibiotic treatment with dispersal cannot eradicate 100% of the bacterial cell population, and the remaining cells can regrow when the antibiotic pressure is removed. Additionally, those surviving biofilms may be enriched in genetic variants with enhanced antibiotic resistance (Drenkard and Ausubel 2002). The selective pressure effect of combined dispersal and antibiotics on biofilms has not tested to date. Thus, the following chapter focuses on whether induction of dispersal at different stages of biofilm formation affects the formation of resistant variants with or without the combination of antibiotic treatment.

Chapter 3: Impact of c-di-GMP Mediated Biofilm Dispersal on the Rate of Tolerant Variant Development

3.1 Introduction

In the previous chapter, c-di-GMP mediated biofilm dispersal has been shown to be a promising strategy to increase antimicrobial efficacy. This strategy, however, remains controversial as several reasons might delay its further usage in therapeutic treatments. First, following dispersal, bacteria might be released from the biofilm into the blood-stream, increasing the risk for acute and life-threatening systemic infections, such as sepsis and severe pneumonia (Fux, Stoodley et al. 2003). Second, “dispersed cells” consist of subpopulations with distinct phenotypes and genotypes, as compared to their planktonic counterparts, which might enhance their virulence mechanisms compared to planktonic cells. Only until recent years, dispersed cells were thought to harbor potentially higher antibiotic resistance than the typical planktonic cells. It has been shown that dispersed bacteria could eventually revert to the planktonic type of cells after multiple generations of growth (Chua, Liu et al. 2014). Finally, environmental selections occurring within the microbial community may result in the release of variant populations with reduced susceptibility to antimicrobial agents. This scenario is especially relevant following prolonged exposure to antimicrobial agents at sub-inhibitory concentrations, as it is often the case during treatments of chronic infections (Wright, Fothergill et al. 2013).

Selection of variants with reduced antimicrobial susceptibility represents a significant threat, as they are typically associated with treatment failure and relapse of infections. Tolerant subpopulations refer to cells with the ability, inherited or not, to survive punctual exposure to high concentrations of antibiotics as a result of a slower metabolism. This phenotype is often observed with carbapenems, which require active cell wall synthesis to kill bacteria (Tuomanen, Cozens et al. 1986). For *P. aeruginosa*, it has been shown, that both stationary-phase bacteria and biofilm bacteria exhibit similar tolerance to antibiotics, and this was primarily attributed to the presence of persister cells (Spoering and Lewis 2001). Tolerant cells exhibit similar MIC values (concentrations required to kill 50% of the populations) as the susceptible parent population, but the MDK₉₉ (MBC or MBEC) is significantly increased (Brauner, Fridman et al. 2016). In contrast, persisters are subpopulations of a clonal bacterial population with the non-heritable ability to survive prolonged exposure to high concentrations of antibiotics without any distinct resistance mechanisms (Lewis, Spoering et al. 2005). Persister cells exhibit similar MIC and MDK₉₉ values as the susceptible parent population, but the minimal duration to eradicate 99.99% of the population, MDK_{99.99}, is significantly higher (Brauner, Fridman et al. 2016).

Biofilms consist of bacterial cells at different stages of the development life-cycle, the population as a whole continually adapts to its surrounding environment. The multitude of mechanisms that enable biofilms to withstand exponentially higher antibiotic concentrations has been collectively termed as “Biofilm recalcitration” (Lebeaux, Ghigo et al. 2014). Although biofilms are considered as the ultimate protection of bacteria, stressful microenvironments

can emerge from the high density of cells existing inside the microbial community. To survive the different micro-niches that characterize this ever-changing environment, microbial cells have to evolve and adapt. These adaptation processes result in genotypic/phenotypic diversification, and several biofilm-derived morphological variants have been previously identified, especially for *P. aeruginosa*. Small-colony variants (SCVs) are the most described morphotype (Häußler, Tummeler et al. 1999, Häußler 2004). SCVs of *P. aeruginosa* have been isolated from both *in-vitro* and *in-vivo* biofilms (Evans 2015). These bacteria exhibit enhanced surface attachment and biofilm formation capabilities, which are believed to facilitate the infection process eventually. Found during the dispersal process in *P. aeruginosa* PAO1 biofilm, this morphotype has been linked to the activity of Pf filamentous phages (Martínez and Campos-Gómez 2016). Other morphotypes include wrinkly variants which are characterized by overproduction of acetylated cellulose and Pel (Lebeaux, Ghigo et al. 2014). Sticky/mucoid variants, characterized by increased production of extracellular alginate or rugose variants which have been shown to auto-aggregate in liquid culture (D'Argenio, Calfee et al. 2002). Morphotype variants have been shown to differ in the affinity to bind the dye Congo-Red, making them easier to identify (Kirisits, Prost et al. 2005, Ueda and Wood 2009).

In the past decades, a particular emphasis has been put on MDR bacterial infections, for which the arsenal of chemotherapies is dramatically diminishing (Izadpanah and Khalili 2015). Colistin is considered the last resort antibiotic against Gram-negative organisms and is routinely used in chronic and difficult to treat MDR bacterial infections, such as cystic fibrosis (CF) (Jansen, Mahrt et

al. 2016). Administered as either mono-treatment or in combinations to yield synergistic potential (Martis, Leroy et al. 2014), resistance to colistin remains seldom (Liu, Wang et al. 2016). Non-heritable and inherited abilities to survive colistin exposures have, however, been reported; the latter being associated to modifications of Lipid-A, its biological target, which is found in the outer Gram-negative membranes (Lee, Park et al. 2016). Due to high fitness costs, mutations are known to revert once colistin stress subsides. Thus, *P. aeruginosa* exhibits transient colistin resistant phenotypes. Lastly, it has been shown that *P. aeruginosa* dispersal through NO shows potential synergy with colistin treatment, in *in-vivo* experiments in a catheter model (Ren, Wu et al. 2016). The similarities between NO mediated dispersal to the p_{BAD} -*yhjH* induced dispersal imply further investigation into increasing the efficacy of this last resort drug.

The objective of this work was to investigate biofilm-associated morphotypes emerging secondary to colistin exposure, and the effect of induced c-di-GMP mediated dispersal might have on their development.

3.2 Methods

Bacterial strains: The strain of *P. aeruginosa* used in this section is PAO1/ p_{BAD} -*yhjH*::GFP; a plasmid carrying *yhjH* under the *araBAD* promoter. The strain also carries a gene encoding for green fluorescent protein (GFP), inserted into PAO1 *w.t* genomic DNA, with miniTn7-Gm^r- p_{pmr} -*gfp* (Chua, Tan et al. 2013). The strain is kept at -80°C and routinely cultured onto LB Agar supplemented with gentamicin sulfate at 60 µg/mL.

Chemical agents: LB Agar, Miller recipe (BD Difco) and MHB or MHA (Sigma-Aldrich), Tryptone, Agar (BD) Congo-red (Sigma-Aldrich) were used. Antimicrobial agents used throughout this chapter were gentamicin-sulfate and colistin-sulfate (MP Biomedicals).

Biofilm growth and quantification of colistin MBEC: Biofilms were grown on 5 mm glass beads (Merck) for 1 to 3 days, as described in chapter 2. Two beads (representing two technical replicates) were placed into each well of a 24-well microtiter plate (NUNC™, Thermo-Fischer). MHB media, containing 0.25% arabinose (Ara+) and non-arabinose containing (Ara-), were used to grow biofilms under constant dispersal induction or without. Every 24 hours, samples were washed with 0.9% NaCl, before replenishing the medium in all tested conditions. Beads were collected daily in 0.9% NaCl; biofilm bacteria were harvested by subjecting the beads to a series of 5 times 10-second sonication (at 37 kHz) and 10-second vortex. Bacterial suspensions were subsequently serially diluted (10^3 - 10^5) in 0.9% NaCl, and 10 μ L aliquots plated on MHA plates containing 0-64 μ g/mL colistin. Following 24-hour incubation at 37°C, CFU/Bead were calculated for each concentration, the colistin MBEC was calculated as the concentration resulting in 99% killing relative to untreated control (that result in $2 \log_{10}(\text{CFU/Bead})$ killing).

Identification of morphological variants: Biofilms were grown for 1-3 days and harvested as described above in the following four experimental conditions:

- MHB containing 0.25% (w/v) arabinose “Ara+ Control”
- MHB containing 0.25% (w/v) arabinose + colistin at 8 µg/mL “Ara+ Colistin”
- Arabinose free MHB “Ara- Control”
- Arabinose free MHB + colistin at 8 µg/mL “Ara- Colistin”

The development of variants due to exposure to antibiotic stress by colistin is evaluated using the Congo-red agar plate assay. 100 µL of each dilution were then spread plated on Congo-red agar plates as previously described (Friedman and Kolter 2004). Congo-red agar was prepared using a solution consisting of an autoclaved solution of 1% Tryptone (10 gr/L) and 1% Agar (10 gr/L). 1 mL of each of the following stock solutions are added: 40 mg/mL Congo-Red, 10 mg/mL $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 20 mg/mL $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (to achieve same salt concentration as in MHB). Variants were selected based on their susceptibility to colistin. A series of Congo-red agar plates containing increased concentrations of colistin (0, 4, 8, 16, 32, 64 µg/mL) were prepared. Plates were then incubated at 37°C for 24 hours and then left for additional 48 hours at room temperature to allow for distinguishable variant growth on the basis of the morphology of the colonies. Experiments were performed 4 times to ensure reproducibility of results (N=4), and thus, each of the follow-up experiments were conducted in 4 biological replicates. Selected variants were then picked with a sterile toothpick, inoculated into 2 mL of MHB media and left 8 hours in a 37°C shaking incubator (200 rpm). Once samples reached similar turbidity at 0.01 O.D₆₀₀, bacterial cultures were used for additional phenotypic assays.

Difference in variant biofilm production: Biofilm formation was quantified using crystal violet (CV, Sigma Aldrich) staining method, as previously described (O'Toole 2011, Chua, Hultqvist et al. 2015). Briefly, 24-well microtiter plates were utilized, 500 μ L of fresh MHB broth were added in each well of 24-well microtiter plates. Each well was inoculated with 500 μ L of the variants grown up to 0.01 O.D₆₀₀. The microtiter plate was incubated overnight at 37°C. The following days, samples were washed and stained with CV (0.01%, vol/vol in dH₂O). After 30 minutes, the excess of CV stain was removed, rinsed with dH₂O, dried off and solubilized in 100% ethanol. The absorbance of each sample was measured at 590 nm using a microplate reader (Infinite Pro 200, Tecan). Three technical replicates were performed for each variant selected and results are presented as mean +/- Standard Deviation (STDEV) (N=4).

Variant antimicrobial susceptibility profile: MIC values were determined using 96-well microtiter plate method, as performed earlier. A gradient of colistin (0-32 μ g/mL) was created in two-fold dilution manner vertically in each plate. Different variants were inoculated in columns 1-12 in duplicates; a total of 6 samples were tested in each plate. Samples were allowed to grow at 37°C. After 24 hours, growth was determined based on turbidity observed following visual inspection and O.D₆₀₀ measurement. The MIC values were defined as the lowest concentrations for which no visible growth was observed or via O.D₆₀₀ measurement if sample turbidity was no more than 3 times the turbidity of control wells.

Variant motility assay: Agar plates were prepared to assay motility (Chow, Gu et al. 2011, Chang, Krishnan et al. 2014). Agar consisted of 0.5% peptone

(5 gr/L), 0.2% yeast extract (2 gr/L) and agar at 0.3% (3 gr/L, for swimming motility), 0.5% (5 gr/L, for swarming motility) and 0.9% Agar (9 gr/L, twitching motility). Following autoclaving, 1% Glucose (w/v, final) was added to the prepared agar before pouring plates in Petri dishes (60 mm standard plates). Inoculation with a sterile toothpick was performed differently for each type of plate: For swarming, the toothpick was placed at the surface of the agar; for swimming, the toothpick was inserted halfway in the agar, whereas for twitching the toothpick penetrated all the way through the agar down to the bottom of the plate. Plates were left in the 30°C incubator for up to 10 hours (swimming), 12 hours (swarming) and 48 hours (twitching). Swimming and swarming plates were imaged directly using the gel imaging station. The GFP fluorescence of cells was utilized to image for better estimation of the colony size as compared to UV which is used in most cases. Images were later analyzed using ImageJ software (<https://imagej.nih.gov/ij>) to calculate the area (in mm²) occupied by bacterial colony on each plate. Twitching motility required careful removal of the agar and CV staining. After proper de-staining of unbound CV by washing with tap water and drying off the plate, a ruler was used to measure the diameter (in mm) of the twitching zone.

Results, plotting and statistical analysis: CFU/Bead numbers on MHA and Congo-red agar plates were collected manually using a mechanical counter; results were collected and computed in Excel 2010. Calculated CFU/bead results and variant development were analyzed for statistical differences and plotted using Prism Graphpad7. Statistical significance was assessed by multiple comparison two-way ANOVA using in software algorithm under conservative Tukey test at 95% confidence interval.

3.3 Results

Colistin MBEC values for different stages of biofilm growth

The bead biofilm system was used to determine PAO1/*p_{BAD}-yhjH::GFP* MBEC of colistin for 1-3 day grown biofilms under continuous induced dispersal (**Figure 3.1**). Untreated biofilm control samples reached cell densities of $>2 \times 10^6$ CFU/Bead, continuous induced dispersal (Ara+) without added colistin did not lead to considerable difference in CFU/Bead numbers (2.3 - 1.9×10^6 CFU/Bead, on average for 1-3 day samples). For 1-day grown biofilms the MBEC of colistin was already at $64 \mu\text{g/mL}$ (2.1×10^3 CFU/Bead $\pm 2.3 \times 10^2$), for the Ara+ 1-day biofilm the MBEC of colistin was between 32 - $64 \mu\text{g/mL}$ (9.3×10^3 - 2.6×10^3 CFU/Bead $\pm 2.25 \times 10^2$). Similarly, for 2-day grown biofilms the MBEC for Ara- samples was $64 \mu\text{g/mL}$ (9.5×10^3 CFU/Bead $\pm 3.6 \times 10^3$), while a sharper drop in CFU/Bead is evident for the Ara+ samples at 32 - $64 \mu\text{g/mL}$ (2.6×10^4 - 2.6×10^3 CFU/Bead $\pm 2.8 \times 10^2$). MBEC for 3-day biofilms is $>64 \mu\text{g/mL}$ for Ara- samples ($3.8 \times 10^4 \pm 7.5 \times 10^3$), (probably 1 dilution); while for Ara+ samples MBEC is still $64 \mu\text{g/mL}$ (1.7×10^4 CFU/Bead $\pm 5.1 \times 10^3$).

Figure 3.1 CFU/Bead count on MHA plates containing 0-64 µg/mL colistin for PAO1/*p_{BAD}-yhjH::GFP* biofilms grown on 5 mm beads for 1,2 & 3 days in MHB with 0.25% arabinose (Ara+) or without (Ara-). Results are from three independent experiments and presented as average values $\pm$ STDEV (N=3).

A clear shift is seen in 3-day biofilms, as higher numbers of resistant population bacteria with 10^4 - 10^5 CFU/Bead are evident at 64 μ g/mL, with constant Ara+ treatment or without. Arabinose treatment does not seem to significantly affect the proportions of antimicrobial resistant subpopulations as no difference was observed in biofilms grown over the course of 3-days between Ara+ and Ara- samples. Consolidating results for planktonic cultures from the previous chapter (**Table 2.1**), the relevant concentrations for PAO1/*p_{BAD}-yhjH* are MIC of 0.5-1 μ g/mL and MBC of 2-4 μ g/mL. Biofilms thus exhibit 4-6 twofold lower colistin susceptibility depending on the maturity of the sample, similar trends to this result are seen in literature (Hengzhuang, Wu et al. 2011).

Variant populations, morphotypes and frequency

Following preliminary experiments, the concentration of 8 μ g/mL of colistin was chosen to represent a sub-optimal antibiotic concentration for continuous exposure to biofilms. It is equal to twice the MBC, is below the MBEC for 1-3 day grown biofilms and is in the range of the average $C_{\max}$ plasma concentration achievable during *in-vivo* treatment (Li, Coulthard et al. 2003).

The Congo-red agar plate method allowed identification of 3 different morphology phenotypes (**Figure 3.2**) and categorized as Smooth, Spreader and Small. Smooth and spreader colonies were large colonies, the latter with a less distinct colony outline. Small colonies were mostly pinkish and tiny.

Figure 3.2 Example for colony morphologies observed on Congo-red containing agar plates, following 24 hours incubation in 37°C and 48 hours at room temperature. In this example a 1-day grown biofilm that was not treated with either arabinose 0.25% or colistin 8 µg/mL ("Control Ara-"). Serially diluted five times (CFU x 10⁶ final), was plated onto Congo-red agar plate supplemented with 4 µg/mL colistin.

Each of these variant populations (smooth, spreader & small) CFU was enumerated and plotted versus the 4 growth conditions (+/- arabinose, +/- colistin 8 $\mu\text{g/mL}$). Variant development was plotted over number of growth days (**Figure 3.3**); using interleaved bar charts over 0-64 $\mu\text{g/mL}$ range of colistin concentration.

Figure 3.3 Development of population variants in $\log_{10}(\text{CFU/Bead})$ (Smooth, Spreader & Small) for 1-3 days of growth, in different growth conditions: MHB only (Control), 8 $\mu\text{g/mL}$ colistin, with or without 0.25% arabinose (Ara+ / Ara-).

X-axis represents colistin concentration in plates [$\mu\text{g/mL}$]. Experiments conducted in quadruplicates, (N=4) expressed as the average value of four independent experiments $\pm$ STDEV.

Results were not normalized to allow for actual CFU/Bead numbers to be conveyed. As shown, at the highest colistin concentration of 64 $\mu\text{g/mL}$, only small colonies growth was evident; the fraction that survives constitutes 0.01% of the population (10^3 - 10^4 CFU/Bead). There were no significant differences in variant development due to different growth conditions for biofilms grown for 1 day up to 3 days. Thus, continued induced dispersal did not cause enrichment for the tolerant small colony morphotype.

Phenotypes of variants

In an attempt to further investigate differences between the variant populations, 20 colonies within the 3 morphotype categories growing on 32 $\mu\text{g/mL}$ colistin plates were selected and tested for various phenotypic assays (colistin MIC, biofilm production, and surface motility); results are summarized in **table 3.1** versus the parent strain (termed “wt” in table).

Although the variants were selected on colistin 32 $\mu\text{g/mL}$ agar, most of the variants (16/20) exhibited increased or similar susceptibility to colistin as compared to the *w.t* population (MIC ranging between <1 to 2 $\mu\text{g/mL}$). When strains were recultured and MIC examined by the broth microdilution methods, it is evident that bacteria outside of the biofilm context become more susceptible.

Table 3.1 Phenotypic properties of 20 selected colonies from the 3 different morphotype categories picked from 32 µg/mL colistin plates.

Phenotype	Colistin MIC [µg/mL]	Biofilm Production CV Abs ₅₉₀ [Relative to wt]	Swimming [mm ²]		Swarming [mm ²]		Twitching [mm]	
wt	1-2	1.00	95.44	+	37.76	+	7	+
Smooth	0-1	1.17	172.05	+	78.46	+	9	+
Smooth	0-1	0.78	133.83	+	35.39	+	15	++
Smooth	0-1	1.46	369.87	++	49.34	+	9	+
Smooth	0-1	1.59	83.17	+	16.87	+	9	+
Smooth	4-8	1.14	133.71	+	30.65	+	10	++
Smooth	4-8	1.35	9.87	-	6.83	+	4	-
Spreader	0-1	0.88	68.54	+	28.51	+	4	-
Spreader	0-1	1.68	53.52	+	14.13	+	6	+
Spreader	0-1	1.57	96.44	+	26.91	+	3	-
Spreader	0-1	1.80	42.12	+	15.82	+	4	-
Spreader	0-1	1.52	125.02	+	324.24	++	2	-
Spreader	1-2	1.30	7.36	-	1.05	-	4	-
Spreader	4-8	1.08	1120.12	++	24.85	+	6	+
Small	0-1	0.64	120.66	+	361.69	++	5	-
Small	0-1	1.17	53.07	+	22.16	+	7	+
Small	0-1	1.80	91.51	+	16.67	+	4	-
Small	0-1	1.29	104.70	+	24.37	+	9	+
Small	0-1	0.69	163.70	+	14.83	+	6	+
Small	1-2	1.18	178.39	+	16.49	+	8	+
Small	4-8	1.39	48.37	+	5.81	-	5	-

Similarly, 17/20 of the tested variants demonstrated increased biofilm production relative to the *w.t.* Examining surface motility; variants demonstrated increased or reduced motility independent of their morphotype. Decreased motility was the most consistent in the Spreaders with 5 out of 7 tested colonies exhibiting reduced swimming, swarming and twitching zones as compared to *w.t* phenotype. Example swimming and swarming motilities phenotypes can be seen in **figure 3.4**.

Figure 3.4 Example motility patterns of selected Smooth, Spreader and Small colony variants originating from the parent PAO1/*p_{BAD}-yhjH::GFP*. Swimming (Left) and Swarming (Right) agar assays have been used to evaluate the motility phenotypes of selected variants, here two examples of each morphotype have been shown. The surface coverage (mm²) has been analyzed using ImageJ software on images acquired in gel imaging station utilizing expressed GFP fluorescence.

The effect of extended growth on variant development

Since no significant differences in the frequency of variants could be identified, more extended duration, preliminary experiment, growing PAO1/*p_{BAD}-yhjH::GFP* under the same treatment groups was conducted. Biofilms were grown for 7 and 12 days and the resulting variant CFU/Bead ratios were compared to 3-day grown biofilms (**Figure 3.5**). Though these results are exploratory at this stage, and more experiments are warranted, it is

clear that the biofilms exhibit higher tolerance to colistin when allowed to grow up to this point. The highest colistin concentration exposure in this experiment, 64 $\mu\text{g/mL}$, resulted only in less than a $\sim 2 \log_{10}$ decrease in CFU/Bead (For instance, 2.7×10^5 CFU/Bead at 64 $\mu\text{g/mL}$ versus 2.1×10^7 CFU/Bead at 0 $\mu\text{g/mL}$, for Ara- Control treatment for 7-day biofilms). The most evident difference between groups occurred in biofilms that were grown for 12 days in Ara+ conditions. When plated on 64 $\mu\text{g/mL}$ colistin, colonies of all three morphotypes could be detected. This result might point at the experimental duration necessary for development and proliferation of biofilm-specific mutations that have higher fitness under colistin stress.

Figure 3.5 Preliminary development of population variants results as $\log_{10}(\text{CFU/Bead})$ (Smooth, Spreader & Small) for 3-12 days of growth, in different growth conditions: MHB only (Control), 8 $\mu\text{g/mL}$ colistin, with or without 0.25% arabinose (Ara+ / Ara-). X-axis represents colistin concentration in plates [$\mu\text{g/mL}$]. For 7-day and 12-day grown biofilms, only one experiment was conducted once with technical duplicates.

3.4 Discussion

C-di-GMP mediated biofilm dispersal has proven to be a promising strategy to increase antimicrobial efficacy *in-vitro*. However, controversy remains on its potential application *in-vivo* (Fleming and Rumbaugh 2017). One of the reasons is the increased risk of sepsis caused by dispersed cells released into the bloodstream. Although these dispersed cells are known to exhibit reduced susceptibility to antimicrobial agents as compared to the susceptible population, they also quickly revert to planktonic mode, suggesting that they do not represent a therapeutic challenge if the bloodstream infection remains manageable. Another reason for the controversy is the potential release of variant morphotypes (Fux, Stoodley et al. 2003, Bjarnsholt, Tolker-Nielsen et al. 2010, Lebeaux, Ghigo et al. 2014). These biofilm-derived morphotypes, selected by environmental stresses as a survival strategy, are of more significant concern as they exhibit increased resistance and/or virulence. The motivation for the present research was to assess whether *in-vitro* induced dispersal together with colistin treatment increases the risk of release of morphotype variants.

The previously described construct of *P. aeruginosa*, PAO1/p_{BAD}-*yhjH* was used as a model organism for proof-of-concept. Phenotypic variants were identified based on their morphotypes on Congo-red agar supplemented or not, with increasing concentrations of colistin (up to 64 µg/mL). Consistent with the literature, 3 distinct colony morphologies were identified and categorized as Small-colony variant, Spreader and Smooth (Kirisits, Prost et al. 2005, Kirov, Webb et al. 2007, Rau, Hansen et al. 2010, Petrova, Cherny et al.

2014). Colony variants have been linked in the literature to differences in production of extracellular polysaccharides: Alginate, Pel and Psl developing in different rates depending on environmental conditions (Rakhimova, Munder et al. 2008, Colvin, Gordon et al. 2011, Mowat, Paterson et al. 2011, Colvin, Irie et al. 2012).

Using biofilms treated for 1-3 days, no statistically significant difference in variant ratio was found when comparing dispersed and non-dispersed biofilms. The factor that contributes most to the variance (>75%) between samples is the colistin concentration in the Congo-red plates, unaffected by arabinose addition or sub-optimal colistin treatment. Therefore, under the current experimental conditions, induction of biofilm dispersal combined or not with colistin exposure, at sub-MBEC does not trigger selection of biofilm variants.

Selected specific morphotype variants were compared to the original strain for motility, biofilm production, and MIC parameters. Evidently, selected variants seemed to revert to phenotypes similar to the *w.t* susceptible population. This was particularly noticeable as the variants were selected on agar containing colistin at 32 µg/mL, but their colistin MIC values ranged from 1 to 8 µg/mL, following sub-culturing. This phenomenon is again consistent with previous reports on colistin variants in Gram-negative bacteria (Moskowitz, Brannon et al. 2012, Workentine, Sibley et al. 2013, Lee, Na et al. 2014); and correlates with the selection of tolerant or heterogeneous resistant cells present initially in the *w.t* populations at very small proportions (Zavascki, Carvalhaes et al. 2010, Miller, Brannon et al. 2011, Hermes, Pormann Pitt et al. 2013).

When examining specific virulence phenotypes, almost all tested colonies tested in the Smooth and SCV morphotypes exhibited increased surface motility behaviors as compared to the *w.t* susceptible strain. Only the Spreaders demonstrated reduced swimming motility, swarming motility and twitching motility behaviors. These results contrast with the literature, which typically reports biofilm variants, and especially SCV, as populations with decreased or impaired surface motility (Harmsen, Yang et al. 2010, Workentine, Sibley et al. 2013, Davies, James et al. 2017). However, it is important to note that identification of SCV variants is extremely challenging, as their morphology highly depends on growth conditions (type of agar, temperature, duration). Furthermore, a SCV on day 1 could turn into a Smooth or Spreader morphotype after few days at room temperature (results not shown). In contrast, Spreader variants were more readily identifiable as their morphology remains the same on selective plates from day 1 and onwards. Motility behaviors and biofilm forming capacity of these variants were consistent with literature; showing reduced surface motility and increased biofilm production as compared to the *w.t*.

In the present study, four independent experiments were performed; two of which were used as the pool for colony selection. 20 colonies were evaluated in total by assessing biofilm production and motility behaviors. Experimental conditions for variants identification represent a significant challenge in this type of research. More accurate observations require stringent protocols to be developed, multiple independent experiments to be performed and numerous variants to be tested. Another limitation of this study is the limited number of virulence phenotypes assessed to characterize the variants. Other phenotypic

assays could have been utilized to better identify the diversification of variants in the population, such as the production of exoproducts (pyoverdine, pyocyanin, rhamnolipids, elastase, proteases) or cell adherence.

Finally, the duration of the experiment may also represent a limitation of this study. A 3-day duration was chosen based on previous findings observed in *in-vitro* flow cell systems (Chua, Yam et al. 2016). However, selection of mutants or irreversible variants from heterogeneous populations under static conditions may require more extended duration. Due to the fact that no significant change to the distribution of morphotypes was evident in the experiment over the course of four repetitions; it was decided to attempt growing biofilms to the point of 12 days. 10 days are often the minimal duration of time necessary to the development of colistin-resistant PAO1 strains *in-vitro*, in static and continuous cultures both (Giacometti, Cirioni et al. 1999, Jochumsen, Marvig et al. 2016, Döbelmann, Willmann et al. 2017).

In an attempt to follow colistin evolution using the experimental protocol developed here, a preliminary experiment performed over 7 to 12 days was performed. Significant changes were observed and might be a possible lead; with regards to the emergence of the different morphotypes: After prolonged exposure at 8 µg/mL for 7 days, an increase in the number of the Spreaders was observed at 32 µg/mL. Furthermore, an increase in CFU/Bead has been observed at the highest concentrations though even in this scenario, c-di-GMP mediated biofilm dispersal once more, did not affect the ratios between the variants.

The differences observed at 12 days were the most evident at the highest concentrations tested. Only at that time point samples that have undergone induced biofilm dispersal had a significant difference to undispersed samples, especially appearance of all morphotypes at 64 µg/mL. It is within this timeframe that colistin resistance can be evolved *in-vitro* for planktonic *P. aeruginosa* (Döβelmann, Willmann et al. 2017). Results suggest that there is potential to evolve biofilm-specific colistin resistant mutants when longer time scales are applied. Unfortunately, due to the complexity of experimental setup and time constraints it was decided to focus on variant generation using the classic technique of serial passages through a gradient of increasing antibiotic concentration (Giacometti, Cirioni et al. 1999).

In conclusion, c-di-GMP mediated biofilm dispersal together with antimicrobial treatment does not increase the risk of development of biofilm-derived variants. These results suggest that c-di-GMP mediated biofilm dispersal could be an interesting strategy at early stages of infections when resistant or tolerant variants have not developed yet as a result of environmental stresses. Further work remains warranted to strengthen the present observations and could include additional experiments with standardized and optimized protocols for identification of biofilm-derived variants.

Chapter 4: Utilization of Induced c-di-GMP Mediated Dispersal as a Strategy to Eradicate Biofilms of Colistin Resistant Strains

4.1 Introduction

Evolution of resistance to antimicrobials is an adaptation of the cell population through mutation and selection. The process is temporal by its nature, as environmental conditions change. Naturally, some mutations that cause increased fitness to overcome specific stress will result in lowered fitness to other stress factors. The principle has been initially shown in Gram-positive *Staphylococcus* species (Sieradzki and Tomasz 1997), where increased resistance to one antibiotic (vancomycin) results in higher susceptibility to other antibiotics such as β -lactams (Barber, Ireland et al. 2014, Dilworth, Ibrahim et al. 2014). This effect, termed “Antibiotic Seesaw Effect”, has frequently been seen as a result of evolved resistance, particularly to the groups of glyco- and lipo-peptides (such as vancomycin and daptomycin, respectively), which target cellular membranes (Vignaroli, Rinaldi et al. 2011, Ortwine, Werth et al. 2013).

Colistin, similar to vancomycin or daptomycin in Gram-positive targeting treatments; is a peptide antibiotic targeting the lipid-A lipopolysaccharide present at the outer cell membranes in Gram-negative pathogens such as *P. aeruginosa* (Lee, Park et al. 2016). Evolution of colistin resistance in *P. aeruginosa* has been shown to occur through mutations in genes of a plethora of two-component systems, such as *phoPQ*, *pmrAB* (Fernández,

Álvarez-Ortega et al. 2013, Lo Sciuto and Imperi 2018) with high redundancy. Mutations always lead to the upregulation of the enzymatic cascade that is responsible for the 4-amino-arabinose modification of Lipid-A, transcribed by the *arnBCADTEF-pmrE* operon (Fernández, Gooderham et al. 2010, Döbelmann, Willmann et al. 2017). Through the action of the enzymes which are the products of the operon, the modifications hide the negative charge of Lipid-A and limit binding of polymyxins (Breidenstein, de la Fuente-Núñez et al. 2011, Miller, Brannon et al. 2011, Lo Sciuto and Imperi 2018). The PhoP/Q and PmrA/B two-component systems, are central signal transduction systems, variations in their expression also affect other cellular attributes, such as virulence, motility and metabolism (Rodrigue, Quentin et al. 2000).

Two-component systems comprise of membrane sensor histidine-kinase proteins, PhoQ and PmrB, which phosphorylate their respective response regulators PhoP and PmrA. The cytoplasmic response regulators, once phosphorylated have an affinity to specific genes and thus modify different cellular response, until dephosphorylated by the kinase protein. The two systems are activated by several signals, most notably, by lower divalent Mg^{+2} cations in the bacterial periplasm (McPhee, Bains et al. 2006). Colistin replaces Mg^{+2} that interact with Lipid-A as a necessary step before binding; thus, activation of the kinases correlates to colistin resistance (Biswas, Brunel et al. 2012).

Resistance to colistin, one of the last resort drugs, is increasing globally at an alarming rate (CDC 2017). Transferrable plasmids carrying *mcr-1*, *mcr-2* and most recently *mcr-3* genes encoding phosphor-ethanolamine transferase

enzymes have been reported worldwide (Denton, Kerr et al. 2002). In different bacterial species (Yin, Li et al. 2017), including *P. aeruginosa* (Lee, Na et al. 2014). Non-heritable and plasmid independent ability to survive prolonged exposure to high concentrations of colistin has also been shown in *P. aeruginosa*, both *in-vitro* and *in-vivo* (Høiby, Krogh Johansen et al. 2001, Moskowitz, Brannon et al. 2012, Döselmann, Willmann et al. 2017, Regenbogen, Willmann et al. 2017, Tan, Vidaillac et al. 2017). Similarly, biofilms of *P. aeruginosa* show increased tolerance to colistin due to the heterogeneity of the cell population and decreased diffusion of the drug (Harmsen, Yang et al. 2010). Furthermore, specific mutations seem to proliferate in biofilm phase more extensively than in the planktonic phase bacteria (Pamp, Gjermansen et al. 2008). This phenomenon is of particular concern, as it represents a significant therapeutic challenge.

In a recent study (Vidaillac, Benichou et al. 2012), unconventional combinations of antimicrobial agents were evaluated as novel alternatives to treat colistin-resistant infections. It was shown that sub-inhibitory concentrations of colistin combined with vancomycin or trimethoprim resulted in a significant synergistic effect against colistin-resistant isolates of *P. aeruginosa*, *Klebsiella pneumonia* (*K. pneumonia*) and *Acinetobacter baumannii* (*A. baumannii*). Vancomycin is a glycopeptide antibiotic that binds to enzymes that catalyze the production of peptidoglycan (Hu, Peng et al. 2016). Vancomycin targets the Lipid II peptidoglycan precursor as it is added to the cell wall, in the periplasmic space. Vancomycin thus has higher efficacy against Gram-positive bacteria, which produce thicker peptidoglycan, hence more targets. In Gram-negative bacteria the existence of the outer membrane

is thought to be the main cause, perturbing vancomycin's access and its killing effect. Certain *E. coli* strains with mutations in genes that effect outer membrane assembly have been found to be more susceptible to vancomycin (Zhou, Kang et al. 2015). Although the mechanisms remain unclear, it is speculated, that colistin resistant variants selected from the heterogeneous susceptible *w.t* population demonstrate increased susceptibility to various antimicrobials, including vancomycin. Following membrane composition changes in resistant populations, it is suspected that they might become susceptible to other antibiotics (Döbelmann, Willmann et al. 2017); in a manner that resembles the "Seesaw Effect". The potential synergy of these combinations in colistin resistant biofilm settings has never been assessed.

Given the promising results observed in the previous chapters, the aims in this section are (1) testing the potential of colistin plus vancomycin combination to treat bacterial biofilms of colistin susceptible and resistant strains, and (2) evaluating the impact of biofilm dispersal on the antimicrobial efficacy of the combination. It is here shown that following induced c-di-GMP mediated dispersal; the seesaw effect is facilitated, enabling a higher synergistic effect between colistin and vancomycin, to eradicate the resistant biofilms. The results demonstrate how the development of resistant bacteria populations could be prevented, and consecutively, once resistant populations develop, the best strategy to eradicate them is based on combinatorial treatment that includes dispersal agents.

4.2 Methods

Bacterial strains: The strain of *P. aeruginosa* used in this section is PAO1/p_{BAD}-*yhjH*; a plasmid carrying *yhjH* under the *araBAD* promoter. This strain is susceptible to colistin (MIC 1-2 µg/mL). An *in-vitro* selected colistin resistant derivative of PAO1/p_{BAD}-*yhjH* was generated through repeated exposure to sub-inhibitory concentrations of colistin; evolution of resistance was conducted in batches, in MHB media, with a stepwise increase in colistin concentration (Giacometti, Cirioni et al. 1999, Cabot, Zamorano et al. 2016). Gentamicin at 20 µg/mL was added to the broth to maintain the plasmid, and initial colistin concentration of 2 µg/mL (twice the MIC) was also added to the initial broth. Tubes were grown in 37°C with shaking at 200 rpm, if following 24 hours, visible turbidity was evident; the sample was used to inoculate a new tube (1:100 dilution) under similar growth conditions at an increased colistin stress. After 14 days of selection, the resistant population was able to grow in 32 µg/mL.

Chemical agents: LB agar, Miller recipe (BD Difco) and MHB or MHA, (Sigma-Aldrich) were used. Antimicrobial agent used for evolution of resistance was colistin-sulfate (MP Biomedicals). Susceptibility was tested versus a total of 25 antibiotic compounds: Colistin sulfate, Polymyxin B sulfate, Ampicillin sodium, Carbenicillin disodium, Neomycin sulfate, Kanamycin sulfate, Streptomycin sulfate, Tetracycline hydrochloride & Rifampin (MP Biomedicals); Penicillin G sodium, Amoxicillin trihydrate, Piperacillin sodium, Aztreonam, Cefsulodin sodium, Ceftazidime hydrate, Imipenem monohydrate, Meropenem trihydrate, Spectinomycin dihydrochloride pentahydrate, Tobramycin, Vancomycin

hydrochloride, Nalidixic acid sodium, Ciprofloxacin, Chloramphenicol, Erythromycin & Sulfadimethoxine (Sigma-Aldrich).

Culture conditions: The parent PAO1/*p_{BAD}-yhjH* strain was routinely cultured onto MHA supplemented with gentamicin sulfate at 60 µg/mL. Cultures were freshly streaked on MHA + gentamicin 60 µg/mL plate prior to further experiments.

Biofilm production: Crystal Violet (CV) staining was used to assess biofilm production as previously described (O'Toole 2011, Chua, Hultqvist et al. 2015). Briefly, 24-well plates containing 500 µL of fresh MHB broth per well were inoculated with 500 µL of the bacterial suspension at 0.01 O.D₆₀₀. Cultures were left overnight to grow at 37°C. Biofilms were then washed with NaCl 0.9%, stained with CV solution (0.01% vol/vol in dH₂O) and solubilized in ethanol. Absorbance at 590 nm was measured using a microplate reader (Infinite Pro 200, Tecan). Experiments were performed in quadruplicates to ensure reproducibility; results are expressed as the average value +/- STDEV (N=4).

Antimicrobial susceptibility assay: MIC values of parent and resistant strains were assessed in MHB as described in previous chapters using 96-well plate broth dilution method (CLSI 2012). Antimicrobials tested are specified under “Chemical Agents” above at concentrations ranging from 0-128 µg/mL. MIC values were defined as the first concentrations for which the turbidity read at O.D₆₀₀ was not higher than 3 times the control wells, supported by unaided visual inspection. The experiment was performed in triplicates (consisting of 2

technical duplicates) to ensure reproducibility; Results are expressed as average values $\pm$ STDEV (N=3).

Growth Curves: Growth kinetics were performed in 24-well plates, containing 500 μ L of fresh MHB broth, inoculated with 500 μ L of the samples at 0.01 O.D₆₀₀. Plates were covered with parafilm to avoid condensation on the lid and incubated at 37°C under shaking conditions (200 rpm). Plates were taken out of the incubator at specific intervals (initially every hour, and at shorter intervals at the logarithmic growth phase) to measure turbidity by O.D₆₀₀ with a microplate reader. Experiments were performed in quadruplicates to ensure reproducibility; results are expressed as average turbidity measurement $\pm$ STDEV (N=4).

Motility Assays: Agar plates were prepared to assess surface motility behaviors as described in the previous chapter. Agar consisted of glucose (1%), peptone (0.5 gr/l), yeast (0.2 gr/L) and agar at concentrations varying from 0.3% (Swimming) to 0.5% (swarming), up to 0.9% (Twitching). Agar plates were inoculated with a sterile toothpick, using single colonies, and incubated at 30°C. Swimming and swarming plates were imaged directly using the gel imaging station by UV fluorescence and images analyzed with ImageJ software (<https://imagej.nih.gov/ij>), to calculate the area occupied by bacteria on each plate in mm. Twitching motility was visualized by staining the biofilm with CV, and a ruler was used to measure its diameter (in mm). Motility assays were performed in triplicates (consisting of 2 technical replicates) to ensure reproducibility; results are expressed as average $\pm$ STDEV (N=3).

Virulence Factors: Production of compounds known to be involved in the virulence of *P. aeruginosa* has been quantified using different biochemical protocols. Inoculums were grown in MHB containing 30 µg/mL gentamycin at 37°C in a shaking incubator at 200 rpm. After O.N. growth, bacterial suspensions were pelleted, washed in NaCl 0.9% and resuspended in MHB up to an O.D₆₀₀ of 0.5. These suspensions were used to inoculate 50 mL MHB in long neck Erlenmeyer flasks with metal caps (O.D₆₀₀ of 0.01) and left to grow O.N. under the same conditions. Sample turbidity was then measured and corrected to O.D₆₀₀ = 1 for normalization of the samples. Bacterial suspensions were then pelleted at 10,000 rpm for 10 minutes. The resulting supernatant was aliquoted for the following procedures.

Pyocyanin quantification: Pyocyanin was extracted as described previously (Frank and Demoss 1959) with modifications. 5 mL samples of the above supernatant were extracted with 1 mL chloroform. 900 µL of the organic phase formed at the bottom of the tubes were pipetted out and transferred into 1.5 mL Eppendorf tubes. The samples were subsequently centrifuged for 1 minute 10,000 rpm to remove impurities. The organic phase (700 µL) was further extracted with 0.1N HCl solution (700 µL). The aqueous phase formed at the top was collected for measurement at 520 nm using a spectrophotometer (UV-Vis, Shimazu). A 0.1N HCl solution was used as a blank. The concentration of Pyocyanine (µg/mL) was estimated by multiplication of Abs₅₂₀ x 17.072 molar extinction coefficient ϵ (El-Shouny, Al-Baidani et al. 2011). The experiment was performed in triplicates to ensure reproducibility, and results are expressed as mean +/- STDEV (N=3).

Rhamnolipid quantification: Difference in rhamnolipid production was determined as described previously (Koch, Käppeli et al. 1991) with additional modifications. Rhamnolipids were extracted from an aliquot of the above supernatant (20 ml) using 5 mL of diethyl-ether. 4 ml of the organic phase formed at the top were carefully pipetted out using a 5 mL pipette and transferred into a 15 mL Falcon tube. Tubes were then centrifuged at 10k rpm for 2 minutes to remove any residues of the supernatant. 2 mL of the transparent top layer were pipetted out and transferred into 2 mL Eppendorf tubes. Samples were left to evaporate overnight in a chemical hood. Residues were then solubilized in 100 μ L dH₂O to which 900 μ L of a solution of 0.19% Orcinol in 53% Sulfuric Acid (H₂SO₄) were added. Samples (including a blank sample containing the stated solubilization buffer only) were heated for 30 minutes using a heat block at 80°C. Samples were then cooled down at room temperature for 15 minutes. Absorption was measured using a spectrophotometer at 421 nm. The experiment was performed in triplicates to ensure reproducibility, and results are expressed as mean +/- STDEV (N=3).

Elastase assay: To assess the levels of extracellular proteolytic enzymes, the Congo-red – elastin assay was used as described (Kessler and Safrin 2014). 2 mL of the above supernatants were filtered through 0.2 μ m filters. 1 mL was pipetted out and transferred into 1.5 mL Eppendorf tubes. Elastin – Congo-red (10 mg) was added to each tube. Samples were vortexed for 30 seconds and incubated at 37°C under shaking conditions (200 rpm) for 16 hours. Following incubation, samples were centrifuged down at 14k rpm for 1 minute, and absorbance was measured at 495 nm using a spectrophotometer. A blank sample was prepared and consisted of MHB plus Elastin – Congo-red.

The experiment was performed triplicates to ensure reproducibility, and results are expressed as mean +/- STDEV (N=3).

Population Analysis Profile (PAP): The presence of subpopulations with varying degree of susceptibility to colistin was evaluated as previously described (Li, Rayner et al. 2006). O.N cultures from both strains were centrifuged 8 minutes at 8K rpm and solubilized to high density ($\sim 10^{10}$ CFU/mL) in MHB. Serial dilutions were prepared in 0.9% NaCl and 100 μ L were spread plated on MHA containing 0, 1, 2, 4, 8, 16, 32, 64 μ g/mL colistin. After 24-hour incubation at 37°C, colonies were counted, and $\log_{10}$ CFU/mL were calculated and plotted versus concentration. Bacteria on plates with higher colistin concentrations demonstrated slower growth and were therefore allowed to develop for an additional 24 hours at room temperature. The experiment was conducted in quadruplicates (N=4) to ensure reproducibility. Hetero-resistance was defined as colonies that grew on plates with colistin concentrations higher than the MIC (1-2 μ g/mL).

DNA extraction, purification, sequencing and analysis: Samples of resistant and parent strains were grown as described above in "Growth Curves". Cultures were harvested at early stationary phase, where 800 μ L of each isolate was pelleted down (10k rpm for 5 mins), and the pellet washed with 0.9% NaCl to remove any residual growth media. QIAamp DNA mini Kit (Qiagen) was used for nucleic acid extraction and DNA purification following the manufacturer's protocol. DNA samples were examined on 1% Agarose Gel in Tris-Acetate-EDTA (TAE) buffer, with a 1 kbp gene ladder (Qiagen). The purity of the DNA was determined by NanoDrop (Thermo-Fischer) and

accurate concentration by Qubit[®] 2.0 Fluorimeter (Thermo-Fischer) following manufacturer protocols. Whole genome sequencing of all the isolates was carried out using Illumina HiSeq2500 platform generating approximately 20 million, 100 bp paired-end reads.

All further bioinformatics analysis was conducted in CLC Genomics Workbench 10.0 (Qiagen Bioinformatics). Quality and adaptor trimming was performed, and any reads below 50bp were discarded. Paired-end reads were then mapped on the PA01 genome (NCBI Accession Number: NC_002516), and variant detection and comparison was carried out. Any SNPs/ nucleotide differences below the frequency of 90% were not processed any further.

RNA extraction, purification, sequencing and analysis: Samples of resistant and parent strains were grown in quadruplicates as described above in “Growth Curves”. Cultures were harvested at early stationary phase, where 600 µL of each isolate (10^8 - 10^9 CFU/mL) were pelleted down (7k rpm for 5 minutes), and pellets washed with 1.2 mL of RNA protector, with vortex for 15 seconds. Mixture Incubated for 5 minutes then Pelleted (7k rpm, 12 minutes) at 4°C. Supernatant completely removed and samples stored at -80°C.

RNeasy Mini-Kit (Qiagen), was used for nucleic acid extraction and RNA purification, based on an on-column DNase digestion principal. The purity of the RNA was determined by NanoDrop (Thermo-Fischer) and accurate concentration by Qubit[®] 2.0 Fluorimeter (Thermo-Fischer), Turbo DNA-free treatment (Ambion, Agilent), was used to degrade residual DNA. Agencourt

RNAClean up XP kit (Beckman-Coulter) magnetic beads were used for RNA clean-up. rRNA depletion performed with RiboZero (Illumina) magnetic beads, RNA integrity was evaluated with Tape-Station (Agilent), samples with excellent RNA Integrity Number (RIN) > 8.0 were processed further. Double-stranded cDNA was synthesized with Next RNA ultra-directional sequencing (NEB) from depleted samples and purification of double-stranded cDNA with Agencourt AMPure XP beads (Beckman-Coulter). Sequencing of cDNA end-product of all isolates (susceptible and resistant strains in three biological replicates) was carried out using Illumina miSeq platform generating 20 million, 300 bp reads. Gene annotation was done using GO (Gene Ontology) annotation (The Gene Ontology 2015).

All further bioinformatics analysis was conducted in CLC Genomics Workbench 10.0 (Qiagen Bioinformatics): Transcript count (reads above 50 bp in size) calculated based on metadata associated with triplicate samples following normalization to their effective size. Statistics are based on a fit to a generalized linear model with a negative binomial distribution, DESeq2 (Anders and Huber 2010). Differentially expressed genes were identified and were determined as differentially expressed when having an absolute fold change >2 and an adjusted p-value <0.05. Results transformed to $\log_2(N+1)$ followed by Heatmap and Principle component analysis (PCA) generation for the differentially expressed genes using d3heatmap and gplot packages in R/Bioconductor (Gentleman, Carey et al. 2004).

Biofilm antibiotic susceptibility assays: Differences in susceptibility between the parent strain ("Susceptible") and the evolved strain ("Resistant") to all

antibiotic compounds listed above. MBIC and MBEC values were determined using the Calgary Biofilm Pin Lid Device (CBD) (NUNC™, Thermo-Fischer) against 1-day biofilms grown in MHB, as described in chapter 3 (Ceri, Olson et al. 1999). A growth plate prepared with inoculums at $5 \times 10^5 - 10^6$ CFU/mL final, placed in the 37°C incubator. Following growth phase, the lid is removed and put in a 96-well wash plate containing 200 µL 0.9% NaCl in each well. Challenge plates were prepared similarly as MIC, for all 25 compounds (0-128 µg/mL gradients), lids transferred to challenge plates and incubated at 37°C for 24 hours. MBIC was analyzed by both eye and O.D₆₀₀ measurement, then MBEC was determined with resazurin REMA assay as described: CBD moved to an “Assay Plate” containing 200 µL MHB media with 60uM final resazurin concentration. Plates were placed in the 37°C incubator for 4 hours, then measured in the microplate reader for fluorescence by excitation at 550 nm and Emission at 600 nm. The experiment was performed in triplicates to ensure reproducibility (consisting of 2 technical replicates); Results are expressed as average values +/- STDEV (N=3).

Biofilm time-kill kinetics: Antimicrobial efficacy was assessed against 3-day-old biofilms formed on the surface of 5 mm glass beads, as described in chapter 2. Two beads (representing two technical replicates) were placed into each well of a 24-well microtiter plate (NUNC™, Thermo-Fischer). Biofilms were next exposed to fresh MHB containing 1% (w/v) arabinose or arabinose free medium (control samples). After 4 hours, samples were treated with colistin at 8 µg/mL and/or Vancomycin at 64 µg/mL. Beads were collected at indicated intervals, subjected to a series of 10-second sonication (at 37 kHz) and 10-second vortex. Bacterial suspensions were subsequently serially diluted in

0.9% NaCl before being drop-plated onto LB agar plates. After 24-hour incubation at 37°C, the residual biofilm was quantified as colony forming units CFU/beads and plotted against time. Experiments were conducted in quadruplicates (2 beads per well x 2 wells per condition). MDK₉₉ was defined as the duration of time necessary to eradicate 99% of the population, relative to controls (Brauner, Fridman et al. 2016). Results are presented as an average CFU/bead from 3 individual experiments (conducted in 2 technical replicates) +/- STDEV.

Confocal Microscopy: The difference in eDNA production between strains was examined using confocal laser scanning microscopy (CLSM, Zeiss 780, Germany). Biofilm samples of both strains were grown on ibidi® 8-well glass bottom μ -slides for 3 days before exposure to colistin at 8 μ g/mL alone or following 4 hours induced dispersal. After 24 hours antibiotic challenge, samples were stained for 30 minutes, using Syto-9 (Live cell stain) at 3.34 μ M and DAPI (Total DNA stain) at 1.44 μ M final concentrations. CLSM further assessed biofilms, Syto-9 (Green channel) excitation with 488-laser line and emission at 499-588 nm and DAPI (Blue channel) excitation with 405-laser line and emission at 410-502 nm. Five images were acquired in Z-stacks for each sample; the experiment was conducted in triplicates (N=3). Image sets were subsequently analyzed for biovolume ($\mu\text{m}^3/\mu\text{m}^2$) using COMSTAT 2 ImageJ plugin (Heydorn, Nielsen et al. 2000) using software auto-thresholding.

Statistical analysis: Data analysis, graph plotting and statistical significance (using student's t-test, multiple t-tests, multiple comparisons one-way ANOVA

& two-way ANOVA, as indicated in figure captions) were conducted in Prism GraphPad 7.

4.3 Results

Phenotypic characterization of colistin susceptible (parent strain) and resistant PAO1/p_{BAD}-*yhjH* derivatives

Significant phenotypic differences were observed between the colistin resistant derivative of PAO1/p_{BAD}-*yhjH* and its susceptible parent strain. **Figure 4.1** illustrates the difference in morphology in both agar and liquid medium. The resistant strain produces more pigment and has a SCV phenotype. Furthermore, there were evident aggregates forming in the tubes, regardless of rigorous shaking at 200 rpm. This phenotype was evident to a varying extent throughout the evolution process.

Figure 4.1 Differences in growth patterns between colistin resistant *P.aeruginosa* PAO1/p_{BAD}-*yhjH* evolved strain (Left) and its parent strain (Middle) on MHA + gentamycin 60 µg/mL plates and in MHB + gentamycin 30 µg/mL (Right) after overnight growth in 37 °C.

Both strains exhibited different growth kinetics, (**Figure 4.2**); when starting from a similar initial inoculum at O.D₆₀₀ at 0.05, the resistant strain remained in lag phase for approximately 2 hours as compared to 1 hour for the susceptible parent strain. Furthermore, the maximal growth in turbidity was lower for the resistant derivative as compared to its parent strain.

Figure 4.2 The two strains exhibit different growth kinetics, when grown in MHB media at 37°C with shaking at 200 rpm. Growth was measured as an increase in turbidity at O.D₆₀₀ in 24-well plates; experiments have been conducted in quadruplicates (N=3).

Striking differences in terms of surface motility behavior were observed between the two related strains (**Figure 4.3**). The resistant strain was swarming and swimming deficient as compared to its parental strain. 100-fold decrease in swimming and 10-fold decrease in swarming motilities could be identified.

Figure 4.3 Differences in swimming (Top) and swarming (Bottom) motility phenotypes between the PAO1/*p_{BAD-yhjH}* parent (left) and resistant strain (right).

Results for all phenotypic assays are summarized in **table 4.1**. Assessment of production of a number of known virulence factors shows that the evolved strain produced between 3-4 times more pyocyanin ($2.37 \pm 0.49 \mu\text{g/mL}$ versus $0.61 \pm 0.19 \mu\text{g/mL}$, for the resistant and parent strains, respectively). 2-3 times more proteolytic enzymes (0.69 ± 0.02 versus $0.24 \pm 0.01 \text{ Abs}_{495}$

in the elastase assay), and also a notable increase in rhamnolipid production (1.16 +/- 0.06 versus 0.77 +/- 0.09 Abs₄₂₁).

Table 4.1 Phenotypic differences between parent and colistin resistant derivative PAO1/p_{BAD}⁻yhjH strains.

Phenotype	Strain	Parent	Colistin Resistant
Biofilm Production	CV [Abs_{590nm}]	1.015 (+/- 0.067)	1.013 (+/- 0.051)
Motility	Swimming [mm²]	681.1 (+/- 80.1)	5.25 (+/- 0.94)
	Swarming [mm²]	35.91 (+/- 8.97)	1.89 (+/- 0.99)
	Twitching [mm]	7 (+/- 1)	3 (+/- 1)
Virulence Factors Production	Pyocyanin [μg/mL]	0.609 (+/- 0.19)	2.373 (+/- 0.495)
	Rhamnolipid [Abs_{421nm}]	0.770 (+/- 0.086)	1.158 (+/- 0.058)
	Elastase [Abs_{495nm}]	0.238 (+/- 0.004)	0.686 (+/- 0.023)

The susceptibilities of the evolved strain versus the parent strain, to various groups of antibiotics, were examined. First, in planktonic phase (MIC) as **table 4.2** recapitulates the results. The evolved resistant strain exhibited 3 two-fold decrease in susceptibility to colistin (MIC of 16 to 32 μg/mL versus 1-2 μg/mL for the parent strain). Mutations and adaptations following the evolution of colistin resistance are not exclusive to colistin alone, but allow resistance to other polymyxins (Polymyxin-B). The resistant strain became more susceptible to all β-lactam antibiotic groups (Narrow & extended spectrum, cephalosporin & carbapenem) in varying degrees (4-7 two-fold) virtually restoring all MICs to physiologically relevant concentrations. The effect is less evident in aminoglycosides except for tobramycin (3 two-fold reduction in MIC). A few

other notable antibiotics that exhibit similar trends are vancomycin and tetracycline (3 two-fold reduction) and rifampicin (4 two-fold reduction).

Table 4.2 MIC values for the PAO1/p_{BAD-yhjH} parent and colistin resistant strains.

Family	Antibiotic	Parent strain MIC (µg/mL)	Colistin Resistant strain MIC (µg/mL)
Polymyxin	Colistin (Polymyxin E)	1-2	16-32
	Polymyxin B	1-2	8-16
Narrow Spectrum β-lactam	Penicillin	>128	16-32
Extended Spectrum β-lactam	Amoxicillin	>128	2-4
	Ampicillin	>128	4-8
	Carbenicillin	16-32	1-2
	Piperacillin	8-16	0.5-1
Monobactam	Aztreonam	2-4	0.125-0.25
Cephalosporin	Ceftsulodin	0.5-1	<0.125
	Ceftazidime	2-4	0.125-0.25
Carbapenem	Imipenem	0.5-1	0.25-0.5
	Meropenem	0.25-0.5	<0.125
Aminoglycoside	Tobramycin	2-4	0.25-0.5
	Neomycin	8-16	4-8
	Kanamycin	>128	64-128
	Streptomycin	>128	>128
	Spectinomycin	>128	>128
Glycopeptide	Vancomycin	>128	16-32
Fluoroquinolone	Nalidixic Acid	16-32	16-32
	Ciprofloxacin	<0.125	<0.125
Naphthacene	Tetracycline	16-32	2-4
Amphenicol	Chloramphenicol	>128	32-64
Rifampicin	Rifampicin	8-16	0.5-1
Macrolide	Erythromycin	32-64	32-64
Sulfonamide	Sulfadimethoxine	>128	64-128

The population analysis profile (PAP) (**Figure 4.4**) of both parent and resistant strains demonstrated heterogeneous resistance to colistin. The susceptible parent strain had a $\sim 1 \times 10^{-6}\%$ detectable sub-population with the ability to survive colistin concentrations of up to 16-32 µg/mL ($10.67 \pm 0.11 \log_{10}$ CFU/Bead for control, versus $3.08-2.88 \pm 0.25$, accordingly). The resistant strain exhibited similar PAP pattern as the parent strain, but the resulting curve

was shifted towards increased concentrations of colistin, with ~1-0.33% of the population capable of growing at 16-32 $\mu\text{g/mL}$ ($9.99 \pm 0.22 \log_{10} \text{CFU/Bead}$ for control, versus $8.02\text{-}7.52 \pm 0.4$, respectively).

Figure 4.4 PAP of parent PAO1/ $p_{BAD}\text{-}yhjH$ strain and its colistin resistant derivative. O.N. culture and serial dilutions plated on MHA containing 0-64 $\mu\text{g/mL}$ colistin. Results calculated as $\log_{10}(\text{CFU/mL})$. The experiment was conducted in triplicates plotted as average values $\pm$ STDEV (N=3).

Genetic characterization of the colistin resistant derivative

DNA from both PAO1/ $p_{BAD}\text{-}yhjH$ strains was extracted and following whole genome sequencing aligned the two genomes against the PAO1 reference genome in the database. A pairwise comparison of the evolved strain's genome versus the parent yielded a significant number of hits indicating a list of point mutations, insertions and deletions. A list of mutations with a 100% frequency is summed up in **table 4.3** with references to literature. Genes encoding TbpA, PhoQ and LpxC demonstrated significant alterations for a total of 6 mutations. Single-nucleotide Variations (SNV) that resulted in the

replacement of a purine (G) by a pyrimidine (T) was observed for both *phoQ* and *lpxC*. In all cases, variations resulted in an amino acid change in coding regions. For *PhoQ*, the mutation led to a probable truncated protein product (nonsense). The gene *tpbA* accumulated the most substantial number of variations as compared to the parent strain. The hyper-mutable hypothetical Pf1 Phage coat protein (Whiteley, Bangera et al. 2001), PA0727, was shown to accumulate the total highest number of 21 mutations, 8 of which in over 90% frequency (91.7-99.9%) in the resistant strain as compared to the parent (Supplementary Table 1).

Table 4.3 Top hits from the pairwise comparison, detected variations between the two PAO1/*p_{BAD}-yhjH* strains at 100% frequency.

#	Locus Tag	Gene Name	Genomic Location	Mutation Type	Position	Mutation	A. Acid Change	Reference
1	PA 1180	PhoQ – Two Component Sensor	1278362 – 1279708 (+)	SNV	1279664	G → T	Glu ₄₃₅ * Nonsense	Jochumsen, et al. 2016 Miller, et al. 2011
2	PA 3885	TpbA - protein tyrosine phosphatase	4350834 – 4351490 (+)	SNV	4351381	C → G	Ala ₁₈₃ Gly	Nicastro, et al. 2014 Ueda, et al. 2009
				SNV	4351393	G → A	Gly ₁₈₇ Asp	
				SNV	4351395	C → A	Leu ₁₈₈ Met	
				MNV	4351387. .4351388	TC → AG	Val ₁₈₅ Glu	
3	PA 4406	LpxC - UDP-3-O-acyl-N-acetyl-glucosamine deacetylase	4938276 – 4939187 (-)	SNV	4938732	G → T	Phe ₁₅₂ Leu	Grégoire, et al. 2017 Boyce, et al. 2014

SNV – Single-nucleotide Variation, MNV – Multi-nucleotide Variation

Gene Expression results:

Extracted RNA from both PAO1/*p_{BAD}-yhjH* strains was used as a template for cDNA sequencing and following filtering, genes were annotated using the GO database. Read counts of all transcriptome triplicates for the susceptible

parent and resistant strains were then performed. Analysis representing the differences in gene expression between replicates is presented in **figure 4.5**.

PCA (**Figure 4.5A**) suggests that ~70% of variance exists in the differential expression results between strains (PCA1), and 13% variance exists between replicates (PCA2). The PCA2 dimension analysis suggested that replicates of the resistant strain were at most twice as varied as the susceptible parent strain. The different levels of expression between strains were evaluated by a negative binomial analysis (**Figure 4.5B**) and was composed of 287 genes satisfying threshold criteria were considered for further analysis, of which, 187 genes were upregulated, and 110 downregulated (**Figure 4.5C**; **Supplementary Tables 2-3**, respectively) in the resistant strain as compared to the susceptible parent.

Figure 4.5 Analysis of RNA sequencing data, of 280 differently expressed genes exhibiting a >2 absolute fold-change (adjusted p-value <0.05) generating (A) principal component analysis (PCA) of triplicates of each strain, parent and colistin resistant and (B) expression heatmap in each strain (Red, upregulation; blue, downregulation), (C) pie chart of gene ontology (GO) categorized by biological function (PseudoCAP).

The gene expressing the highest log₂ fold-change (8.27) is PA3559 *pmrE*, which is part of the *arnBCADTEF-pmrE* operon (Fernández, Álvarez-Ortega et al. 2013, Olaitan, Morand et al. 2014). 9 additional genes in the operon were detected in similarly high expression levels (log₂ fold-change of 8.21-6.59). The three constituents of the *oprH-phoP/Q*, which take part in the two-component system that controls the expression of the *arn* operon were the next to be detected in high levels (log₂ fold-change of 6.24-5.26). The hypothetical proteins PA4517, PA4010, PA4011 and were also upregulated (log₂ fold-change 4.65, 4.19, 3.89, respectively), known to be under the control of *phoPQ* and *pmrAB* (McPhee, Bains et al. 2006) and related to reduced divalent cation concentrations near bacterial membranes. Mutations in *tpbA* culminated to higher expression of the gene as well (log₂ fold-change 4.65).

The genes PA3441-7, who were shown in related *Pseudomonas* species to be part of the cell sulfur transport mechanism (*sus* operon) (Scott, Hilton et al. 2007) were the top downregulated genes in this analysis (log₂ fold-change -3.63, -2.75, -2.89, -1.28, -2.25, -1.78 respectively). The gene *tauD*, taurine dioxygenase, which is regulated by *sus* was also found downregulated (-2.29). Generally, different systems related to catabolism of different compounds were the main to be downregulated.

Biofilm characterization

Differences in antimicrobial susceptibility between biofilms of the 2 strains were assessed using the CBD, as described above. MBIC and MBEC values for 1-day-old biofilms are shown in **table 4.4**.

Table 4.4 MBIC and MBEC range values for both PAO1/p_{BAD-yhjH} strains, 1-day grown biofilms.

Family	Antibiotic	Parent Strain		Colistin Resistant Strain	
		MBIC (µg/mL)	MBEC (µg/mL)	MBIC (µg/mL)	MBEC (µg/mL)
Polymyxin	Colistin (Polymyxin E)	4-8	8-16	16-32	32-64
	Polymyxin B	8-16	8-16	8-16	8-16
Narrow Spectrum β-lactam	Penicillin	>128	>128	>128	>128
Extended spectrum β-lactam	Amoxicillin	>128	>128	64-128	64-128
	Ampicillin	>128	>128	64-128	64-128
	Carbenicillin	8-16	>32	8-16	16-32
	Piperacillin	64-128	64-128	8-16	16-32
Monobactam	Aztreonam	>128	>128	>128	>128
Cephalosporin	Ceftsulodin	>32	>32	>32	>32
	Ceftazidime	>32	>32	>32	>32
Carbapenem	Imipenem	2-4	4-8	0.5-1	0.5-1
	Meropenem	2-4	4-8	0.5-1	0.5-1
Aminoglycoside	Tobramycin	0.25-0.5	8-16	1-2	2-4
	Neomycin	16-32	32-64	16-32	16-32
	Kanamycin	>128	>128	>128	>128
	Streptomycin	>128	>128	>128	>128
	Spectinomycin	>128	>128	>128	>128
Glycopeptide	Vancomycin	>128	>128	64-128	>128
Fluoroquinolone	Nalidixic Acid	32-64	32-64	32-64	32-64
	Ciprofloxacin	1-2	1-2	0.5-1	1-2
Naphthacene	Tetracycline	32-64	32-64	16-32	32-64
Amphenicol	Chloramphenicol	>128	>128	>128	>128
Rifampicin	Rifampicin	16-32	16-32	4-8	16-32
Macrolide	Erythromycin	>128	>128	64-128	64-128
Sulfonamide	Sulfadimethoxine	>128	>128	>128	>128

Differences in susceptibility to colistin were noticed with MBIC and MBEC values of 4-8 and 8-16 µg/mL, respectively for the parent strain versus 16-32 and 32-64 µg/mL for the resistant strain. It is noticeable that the same mechanisms that can be attributed to resistance development in planktonic cultures are not the only effectors when evaluating *P. aeruginosa* biofilms. There were no striking differences in MBIC and MBEC values between the parent and resistant strains for most antibiotics, excluding a few. A consistent drop in resistance for both carbapenems (imipenem and meropenem) from

MBIC of 2-4 µg/mL and MBEC of 4-8 µg/mL for the parent strain to MBIC and MBEC of 0.5-1 µg/mL for the resistant strain. For tobramycin, from the MBIC value of 0.25-0.5 µg/mL for the parent strain, the resistant strain has become less susceptible, up to 1-2 µg/mL. However, the MBEC for the resistant strain has decreased, from 8-16 µg/mL for the susceptible to 2-4 µg/mL. These results support the results in chapter 2: Imipenem and tobramycin exhibit high efficacy potential in mono-treatment against young biofilms. Rifampicin and piperacillin exhibit 3 two-fold drops in susceptibility: the parent strain shows MBIC and MBEC values for rifampicin at 16-32 µg/mL, and the resistant strain shows a similar MBEC, but a drop to 4-8 µg/mL in MBIC is evident. Piperacillin shows a drop from 64-128 µg/mL MBIC and MBEC values for the parent to 8-16 and 16-32 µg/mL MBIC and MBEC values for the resistant.

Difference in EPS eDNA production between parent and resistant strains

TpbA is a sensor phosphatase protein negatively regulating the DGC TpbB (Pu and Wood 2010), loss of TpbA has been shown to cascade in increased c-di-GMP production, through TpbB, leading to increased EPS formation (Ueda and Wood 2009, Harmsen, Yang et al. 2010); and to a decrease in eDNA production (Ueda and Wood 2010). Mutations in TpbA were shown to result in both reduced eDNA content, and higher intracellular c-di-GMP. Because the *tpbA* gene accumulated the highest number of mutations in the resistant strain (**Table 4.3**), it was hypothesized that it would lead to a decrease in eDNA production, by increasing c-di-GMP.

The roles of eDNA in colistin resistance are thought to be double: As a chelator for both the drug and Mg^{+2} , reducing colistin diffusivity and activity but

also inducing PhoP/Q (Mulcahy, Charron-Mazenod et al. 2008). To further understand the eDNA production differences between parent and resistant strains CLSM was utilized on 3-day grown biofilm bacteria of both strains. It was hypothesized that induced dispersal by *yhjH*, while reducing intercellular c-di-GMP, would also increase eDNA/Bacteria ratios.

The combined effect of 1% arabinose induction with 8 µg/mL colistin was further evaluated. Biofilms were stained with two dyes: Syto9 (Green) that stains “live cells”, and DAPI, which stains all DNA in the sample. The ratios of DAPI to Syto9 fluorescence were calculated for each treatment of each strain (**Figure 4.6**). No difference was observed in the control samples (Control Ara-) between the resistant and parent strains (dead/live ratio: 1.49 +/- 1.33 versus 1.92 +/- 1.44 DAPI/Syto9, respectively) (**Figure 4.6B**). Following arabinose induction, a decrease in DAPI/Syto9 was observed in the resistant strain treated with colistin as compared to the untreated control (0.53 +/- 0.41 versus 1.49 +/- 1.33, $p < 0.05$). Two-way ANOVA analysis defined the difference between strains as the most significant independent variable contributing to variance in DAPI/Syto9 ratios (~12%, $p < 0.0001$). Assuming higher intracellular c-di-GMP facilitates lower colistin susceptibility in the resistant strain; arabinose induction of c-di-GMP mediated dispersal is thought to revert this effect, increasing colistin susceptibility.

Figure 4.6 CLSM images (20x objective) **(A)** of 3-day-old biofilms of PAO1/*p_{BAD}-yhjH* colistin resistant (Top) and parent (Bottom) strains treated with arabinose 1% (Ara+) or untreated (Ara-) for 4 hours followed by 24 hours exposure to colistin at 8 μ g/ml or not (Control). Biofilms were stained with Syto9 and DAPI prior imaging. Cells appear green; total DNA appears blue and cyan when co-localized. Scale bar is 20 μ m. **(B)** Bacterial load and total DNA after 24-hour antibiotic exposure reported as DAPI/Syto9. Images from five independent experiments (N=5) were analyzed to determine dead/live ratios for each condition, presented as average value $\pm$ STDEV. Results analyzed to evaluate eDNA/Cells ratios for each condition in both strains. Statistical significance analysis conducted using two-way ANOVA, corrected by Sidak hypothesis testing (p-value interpretation: * <0.05).

Antimicrobial killing efficacy of colistin and vancomycin against 1-day-old biofilms

The potential for the synergy of the combination of colistin and vancomycin at 0.25 of the MBEC (8 and 64 $\mu\text{g/mL}$, respectively) was evaluated against 1-day-old biofilms with or without induced c-di-GMP mediated dispersal (Figure 4.7).

Figure 4.7 C-di-GMP mediated biofilm dispersal induced by arabinose allows synergistic antimicrobial effect for colistin combined with vancomycin against the resistant PAO1/*p_{BAD}-yhjH* but not its parent strain. Residual bacterial load $\log_{10}(\text{CFU/Bead})$ after exposing 1-day-grown biofilms of both strains. Biofilms were treated (Ara+) or untreated (Ara-) with 1% arabinose for 4 hours and then exposed to colistin (8 $\mu\text{g/mL}$) or vancomycin (64 $\mu\text{g/mL}$) alone or in combination (at time=0). Experiments conducted in quadruplicates (N=4).

Without induced dispersal, the antibiotic combination resulted in a substantial drop in $\log_{10}$ CFU/Bead in the resistant strain following 8 hours of exposure compared to control (5.74 versus 7.38). With c-di-GMP mediated dispersal, there was a further drop in values (5.08 versus 6.4), but the drop in the control can mean an additive effect of dispersal itself. In contrast, at the 24-hour time point, resistant strain samples following induced dispersal exhibited a sustained 2 $\log_{10}$ drop in CFU/Bead under the combination (5.15 versus 7.46); in the non-dispersed resistant strain, biofilms could recover (6.36 versus 7.5 in the control).

In the parent $p_{BAD^-}yhjH$ strain in the samples with induced dispersal after 4-8 hours, colistin treatment alone results in a drop to 5.36-5.39 $\log_{10}$ CFU/Bead versus controls (6.91-6.96) in the respective time points. Combined antibiotic treatment in these samples resulted in a further decrease at 4-8 hours (5.12-5.24). At the 24-hour time point, both colistin and colistin plus vancomycin treatments contained 6.83-6.82 $\log_{10}$ CFU/Bead versus 7.4 in the control. The values at the 8-hour time point following dispersal reveal that, as expected, the resistant strain was more susceptible to vancomycin (5.57 $\log_{10}$ CFU/Bead) and the parent strain was more susceptible to colistin (5.39 $\log_{10}$ CFU/Bead). It is evident then that the MDK₉₉ by this combination at these antibiotic concentrations lies between 8-24 hours, but only following induced dispersal.

4.4 Discussion

Over 80% of infections are biofilms associated (Burmølle, Thomsen et al. 2010), at the majority of cases biofilms are comprised of multiple pathogenic species, with a various antimicrobial susceptibility pattern. Combination therapy is the most common and efficient strategy to treat these infections (Høiby, Bjarnsholt et al. 2010). However, when infections involve MDR pathogens, such as a colistin resistant *P. aeruginosa* used here, treatment options are limited. One research avenue to combat resistant species is to investigate unconventional strategies, combining drugs which at first sight should not retain any activity against the pathogen (Buyck, Tulkens et al. 2015). These unconventional combinations are typically designed based on predicted mechanisms of resistance. For example, unusual treatment combining daptomycin with trimethoprim was found to be an efficient therapeutic alternative both *in-vitro* and *in-vivo* against daptomycin resistant *Staphylococcus aureus* (*S. aureus*) (Avery, Steed et al. 2012, Steed, Werth et al. 2012). In the same spirit, a combination of colistin and vancomycin was found to be efficient *in-vitro* against colistin resistant strains of *P. aeruginosa*, *K. pneumonia* & *Acinetobacter baumannii* (*A. baumannii*) (Vidaillac, Benichou et al. 2012). The latter has however never been proven efficient against biofilms, which are the most common type of infections.

The objectives of this study were to 1) assess the potential for synergy of the unconventional antimicrobial combinations and 2) evaluate the potential of c-di-GMP mediated biofilm dispersal to increase antimicrobial efficacy; against biofilms of colistin susceptible and resistant strains of *P. aeruginosa*. Dispersal

of biofilm utilizing this specific cellular mechanism has proven to be a promising strategy to treat biofilms of susceptible strains (Roizman, Vidaillac et al. 2017). The question here is whether this strategy is effective against biofilms of resistant bacteria. More specifically, the potential of c-di-GMP mediated biofilm dispersal was evaluated as a strategy to increase antimicrobial efficacy and eventually reach bactericidal activity against biofilm of MDR *P. aeruginosa*. For the purpose of this study, a pair of susceptible and colistin resistant PAO1/p_{BAD}-yjhH strains was used as a proof-of-concept.

A colistin resistant derivative of PAO1/p_{BAD}-yjhH was generated through repeated exposure to increasing sub-inhibitory concentrations of colistin in liquid batch over 14 days, as known from the literature (Cabot, Zamorano et al. 2016). The resulting resistant strain exhibited 5 two-fold decrease in susceptibility to colistin as compared to the parent strain, with a MIC of 32-64 µg/mL versus initial MIC of 1-2 µg/mL (**table 4.2**). Several phenotypic differences have been shown between the parent and resistant strains following the evolution of colistin resistance (**table 4.1**). An increased production of pigment during growth in MHB was noticed, and estimated an increase of up to 4 times in the concentration of pyocyanin in the solution. This phenomenon has been reported in the literature before, following *in-vivo* exposure to sub-inhibitory concentrations of antibiotics, including colistin (Wright, Fothergill et al. 2013). Additional increase in known virulence factor production, rhamnolipids and proteolytic enzymes, has also been detected; as well as a significant 100-fold decrease in swimming and 10-fold in swarming motilities, all correlate to colistin resistance development (Finlayson and Brown 2011). It was noticeable that the resistant strain tends to aggregate even

during growth in liquid batch and grow as a SCV on agar. This is in line with the increase in surface attachment and/or aggregation seen as a result of antibiotic exposure (Hogardt and Heesemann 2010, Thien-Fah Mah 2012, Moradali, Ghods et al. 2017). Colistin stress has thus selected out of the natural heterogenic resistance in the population, both biofilm forming and virulent, SCVs. The higher intracellular c-di-GMP concentration necessary to promote this phenotype might explain the higher effect dispersal via this specific mechanism had on the colistin resistant PAO1/p_{BAD}-*yhjH* versus the parent strain.

Whole genome sequencing and pairwise comparison of the evolved resistant strain versus the parent indicated that a significant number of genetic variations had been selected. Genes encoding the proteins PhoQ, TpbA and LpxC demonstrated the most significant alterations to 100% of sequences. PhoQ is a sensor kinase protein part of the two-component system PhoP-PhoQ (Gellatly, Needham et al. 2012). This system has been linked to cationic antimicrobial resistance before and especially to polymyxin resistance (Gooderham, Gellatly et al. 2009, Gutu, Sgambati et al. 2013). PhoQ/P system controls the enzymatic cascade operon *arn* that modifies the bacterial Lipid-A, an essential constituent of the bacterial membrane and the target for polymyxins (Miller, Brannon et al. 2011). *In-vivo P. aeruginosa* isolates from CF with variations in the PhoQ/P system have been described (Gellatly and Hancock 2013, Mustafa, Chalhoub et al. 2016). Variants are reported to have lower motility, higher virulence, and are correlated to increase in inflammatory reactions. An identified nonsense mutation in Amino Acid 435 (Original Glu₄₃₅), leading to a premature stop, which might have resulted in a 14 amino acid

shorter truncated version of PhoQ to be translated. A structural study of PhoQ in *E. coli* has demonstrated that some mutations in this cytoplasmic domain can lead to increased activity (Marina, Mott et al. 2001).

The periplasmic domain of PhoQ interacts with Mg^{+2} cations via Glutamic acid residues (Prost, Daley et al. 2007). Colistin replaces Mg^{+2} cations in the interaction with Lipid-A, a necessary step prior to its incorporation in to the membrane causing bacterial cell leakage (Biswas, Brunel et al. 2012). PhoQ is activated by lower Mg^{+2} concentrations, it phosphorylates PhoP which directly activates translation of the *arnBCADTEF-pmrE* operon (Fernández, Gooderham et al. 2010, Olaitan, Morand et al. 2014); PhoP also autoregulates its own *oprH-phoP-phoQ* operon and another two-component system PmrA/B, which also increases expression of the *arn* operon. Differential expression results presented here indeed showed increased expression of these abovementioned systems. Overexpression of *arn* is known to provide the highest levels of innate resistance to polymyxins in *P. aeruginosa* (Fernández, Álvarez-Ortega et al. 2013, Lo Sciuto and Imperi 2018); and is achieved by high redundancy of controlling two-component systems.

LpxC is the first enzyme in the cascade that catalyzes the synthesis of Lipid-A, and it is the target for many new tentative bacterial inhibitors (Mdluli, Witte et al. 2006, Tan, Vidaillac et al. 2017). LpxC catalyzes the transformation of UDP-glucuronic acid to 4-amino-arabinose addition (Johnson, Nation et al. 2017). The mutation discovered results in a non-synonymous amino acid change Phe₁₅₂Leu possibly resulting in decreased production of Lipid-A, as

described before (Jochumsen, Marvig et al. 2016). Moreover, it would lead to increased virulence by bacterial endotoxins (Gough, Hancock et al. 1996).

The gene that showed the highest number of detected variations was *tpbA*, the product of this gene, a tyrosine phosphatase that normally negatively regulates TpbB. TpbB is a DGC that has been linked to increased eDNA production, via increased intracellular c-di-GMP concentration (Ueda and Wood 2010). Mutations in *tpbA* have been shown to result in an increase of c-di-GMP, due to TpbB activity, and lead to increased production of EPS polysaccharides and SCV phenotype (Ueda and Wood 2009, Pu and Wood 2010). Here, it was shown that no significant changes between parent and resistant strains. A relative change to eDNA production and decrease in the ratio of eDNA/bacteria was observed following 24-hour antibiotic challenge, but remains of low significance. The effect of eDNA on colistin susceptibility is thought to occur through the chelation of Mg^{+2} of which colistin killing mechanism is dependent (Mulcahy, Charron-Mazenod et al. 2008); it is also known that this effect is negated at Mg^{+2} rich media such as MHB media.

The original experimental plan for CLSM in this chapter was to estimate eDNA and Psl ratios versus live cells in the parent and derivative strains. DAPI was used for staining total DNA (blue channel), SYTO9 for live cells (green channel) and the Psl binding fluorescent stain Texas-Red[®]-conjugated Concanavalin (Molecular Probes, USA) was used in the red channel. Unfortunately, the protocol to use the latter Psl stain could not be optimized for this experiment based on 8-well slides, but did not hinder the staining and analysis of the former two channels. Recently this stain was successfully

applied to *P. aeruginosa* using an *in-vitro* “slide biofilm” model (Chin, Sinha et al. 2017), and is still believed to be useful to attempt to repeat it for this set of experiments. Considering that eDNA is one of the most important negatively charged constituents of the EPS, a decrease in its production relative to Psl would cause the EPS to become more of a cationic nature, possibly inhibiting colistin diffusion based on an electric charge (Batoni, Maisetta et al. 2016). The hypothesis is that higher intracellular c-di-GMP will lead to more biofilm and EPS formation (Harmsen, Yang et al. 2010), with lower eDNA content (Ueda and Wood 2010), better protect from polymyxin stress. Apart from exemplifying the ability of *P. aeruginosa* to adapt to changing environmental conditions and stresses, it is necessary to perform more experiments to describe the connections between c-di-GMP mediated dispersal and antibiotic susceptibility better.

The genetic profile and differential gene expression evident here have been described in detail in the literature (Moskowitz, Ernst et al. 2004, Fernández, Gooderham et al. 2010, Moskowitz, Brannon et al. 2012). Colistin resistance, has been shown to be multi-component (Jochumsen, Marvig et al. 2016): mutations in genes of *PhoPQ*, *pmrAB* & *lpxC* systems lead to upregulation of the *arnBCADTEF-pmrE* operon (Gunn, Ryan et al. 2000, Barrow and Kwon 2009). Additionally, it has been shown that mutations in only mechanisms *pmrAB* or *phoPQ* by themselves were not sufficient for the highest resistance phenotype evident (Jochumsen, Marvig et al. 2016). Rather than a redundant effect, differential expression and upregulation of both systems was shown to be complementary. Recently, a morbidostat, continuous culture system was used to examine the kinetics of accumulation of mutations and differential

gene expression in *P. aeruginosa* clinical isolates from colistin treated CF patients (Regenbogen, Willmann et al. 2017). The results concur with gene expression results shown in this work, mutations in *PhoPQ* and *pmrAB* systems lead to the higher expression of the *arn* operon. *P. aeruginosa* exhibits a heterogenic resistance to colistin initially, the evolution of resistance to higher concentrations of colistin occurs following 7-12 days of drug exposure, in biofilms and planktonic cultures; consistent with evidence from chapter 3, and in this chapter.

Considerable changes to the MIC profile are evident when comparing the colistin resistant PAO1/p_{BAD}⁻*yhjH* and its parent. Development of resistance to one polymyxin (colistin) has resulted in an increase of resistance to another polymyxin antibiotic, showing, at least in part, that resistance mechanisms apply to every group of antibiotics, once resistance to one is established (Olaitan, Morand et al. 2014). The consistent increase in susceptibility to virtually all β -lactam antibiotics tested has proved the existence of the “Antibiotic Seesaw Effect”, in this strain as a direct result of colistin exposure. In a similar manner to what has been shown in Gram-positive organisms, the emerging resistance to drugs which target the bacterial membrane and cell wall, such as daptomycin and β -lactams (Vignaroli, Rinaldi et al. 2011). Structural changes to these cellular barriers enable a considerable increase in antimicrobial efficacy for one group when resistance has developed for the other, and vice versa (Ortwine, Werth et al. 2013). The structural similarity and mechanisms of action between daptomycin and colistin necessitate the “Antibiotic Seesaw Effect” to be taken into account when developing potential solutions for colistin resistance. Furthermore, it was shown here that a

considerable drop in resistance of *P. aeruginosa* to vancomycin, an antimicrobial drug used to treat Gram-positive infections becomes feasible using the “Seesaw Effect”.

The effect of the biofilm mode of growth in regards to susceptibility has been substantial; the differences in MIC were almost wholly diminished, except for a handful of antibiotics (**Table 4.4**). According to results, carbapenems, tobramycin, piperacillin and rifampicin are shown to be the most promising treatments within the antibiotics tested to tackle colistin resistance. The potential for synergy of vancomycin and colistin against the parent and resistant strains was shown as a promising strategy for planktonic cultures (Vidaillac, Benichou et al. 2012). Here, the “seesaw effect” was not observed in biofilms initially; it was shown here via biofilm time/kill analysis, that synergy between colistin and vancomycin is only possible following induced c-di-GMP mediated dispersal (**Figure 4.7**). This demonstrated the potential of biofilm dispersal as a therapeutic strategy to prevent and eliminate the development of colistin resistance in *P. aeruginosa*, the suggested mechanism to this synergy is presented as a scheme in **figure 4.8** below.

The main limitation of this research is the small number of strains, a parent, and a resistant PAO1/*p_{BAD}-yhjH* strain. There is a possibility that the effects that shown here are limited to *P. aeruginosa*, and it cannot be ruled out that the concept of c-di-GMP mediated dispersal would involve other factors in other species. However, it was already reported that a stronger synergistic effect of the vancomycin and colistin combination against *K. pneumonia* and *A. baumannii*, two major human pathogens (Vidaillac, Benichou et al. 2012).

Further research is now warranted to confirm the potential of c-di-GMP mediated biofilm dispersal and vancomycin combination with colistin against biofilms of both pathogen species.

In conclusion, colistin resistance in *P. aeruginosa* seems to result in increased susceptibility to vancomycin, which is reminiscent of the “Seesaw Effect” described in Gram-positive bacteria. Data analysis suggests that synergy may result from heterogeneous colistin resistance, in both colistin resistant and parent strains. Nonetheless, the colistin resistant population demonstrates increased susceptibility to vancomycin (among other antibiotics) and is enriched as compared to the colistin susceptible strain. In the presence of both drugs, colistin will target the colistin susceptible population, which is resistant to vancomycin, and on the other hand, vancomycin will kill the colistin resistant population that demonstrates an increased susceptibility to vancomycin. Dispersal is instrumental in utilizing the seesaw effect in biofilm bacteria, as it facilitates access to both drugs to the different populations. The seesaw effect observed in both Gram-positive and Gram-negative bacteria should be further investigated as it has a high potential for treatment. An attempt to develop a dispersal treatment via c-di-GMP depletion, based on this proof-of-concept study is both plausible and essential.

Figure 4.8 Schematic description of the combined effect of c-di-GMP mediated biofilm dispersal and the "seesaw effect". **1a.** Colistin exposure and sub-optimal concentrations result in enrichment of the colistin resistant sub-population. **1b.** Resistant subpopulations become more susceptible to vancomycin. **2a.** Biofilm dispersal is crucial to enable penetration of both drugs through the EPS. **2b.** Combined vancomycin and colistin treatment following dispersal has the potential for bactericidal effect.

Conclusions and Future Prospects

Bacterial biofilms are considered difficult to treat due to (1) slow or impaired penetration of antimicrobials in their EPS matrix and (2) the presence of different sub-populations existing at different metabolic states and exhibiting different susceptibility profiles within the same community. Antimicrobial combinations have been proven to be the recommended strategy to treat biofilms as they exhibit superior efficacy as compared to mono-treatments (Černohorská and Votava 2008, Herrmann, Yang et al. 2010, Høiby, Bjarnsholt et al. 2010, Hengzhuang, Wu et al. 2011). Despite the increased use of combined treatments, antimicrobial killing efficiency often remains limited. Novel research is focused at the development of adjunct therapy capable of increasing the efficacy of synergetic compounds. Over the past decades, numerous studies have suggested the enormous potential of dispersal, by various means, to eradicate biofilms (Banin, Brady et al. 2006, Landini, Antoniani et al. 2010, Barraud, J. Kelso et al. 2015).

In this thesis, c-di-GMP mediated biofilm dispersal was evaluated as a therapeutic strategy to enhance antimicrobial efficacy against *P. aeruginosa* biofilms. Proof-of-concept studies were designed and successfully achieved to demonstrate that (1) c-di-GMP mediated biofilm dispersal enhances antimicrobial synergy, (2) does not increase the risk for resistant variant selection and (3) is crucial for achieving an efficient killing effect which are attainable, even when MDR strains are involved.

First, using a *P. aeruginosa* laboratory construct strain, PAO1/p_{BAD}-*yhjH*, carrying the PDE gene inducible upon addition of arabinose. The induced drop in intracellular c-di-GMP resulted in a maximum drop of 1 log₁₀ CFU/Bead, significant, but the majority of the biofilm population remains and regrows even under consecutive expression. It was demonstrated that the synergistic combination of imipenem and tobramycin is enhanced following *YhjH* induction in mature biofilms. The induction of *YhjH* must hamper antimicrobial biofilm tolerance mechanisms, which are c-di-GMP regulated (Ha and O'Toole 2015); significant and sustained killing effect was achieved *in-vitro* at physiological relevant antimicrobial concentrations only following induction.

To further evaluate the potential of c-di-GMP mediated dispersal as a possible therapeutic strategy, the risk for development of variants or sub-populations was assessed with tolerant or resistant phenotypes. Biofilm variants emerging during or following *in-vitro* or *in-vivo* colistin therapy is a well-described phenomenon (Wright, Fothergill et al. 2013, El-Halfawy and Valvano 2015, Brauner, Fridman et al. 2016, Lee, Park et al. 2016, Regenbogen, Willmann et al. 2017). It has been shown *in-vivo*, that in many clinical conditions, such as in CF patients lung sputum, the phenomenon is caused by the inability to reach adequate colistin concentrations (Beringer 2001, Moskowitz, Brannon et al. 2012, Lee, Na et al. 2014, Mustafa, Chalhoub et al. 2016, Grégoire, Aranzana-Climent et al. 2017). Using colistin as a model treatment, this work demonstrated that the emergence and morphotypes of variants are similar in dispersed and non-dispersed biofilms. However, preliminary results suggest that the minimal duration for experimental colistin resistance evolution *in-vitro* should be extended. The minimum reported time until resistance development

in planktonic cultures is 12-14 days (Lee, Park et al. 2016). The “Bead biofilm” protocol was utilized here for time/kill experiments and variant selection. It allowed for reproducible samples of biofilms as shown here for *P. aeruginosa* at 10^7 CFU/bead, in high-throughput manner. A future experiment, employing this system for AMR phenotypes, variant selection, also by repeated sub-inhibitory exposure technique, should be considered. Differential gene expression of the biofilm forming fraction in samples and possible biofilm-specific mutations are foreseeable.

The potential of c-di-GMP mediated biofilm dispersal was further evaluated against biofilms of MDR strains. A colistin resistant derivative of our construct PAO1/p_{BAD}-*yhjH* was generated. Of interest and consistent with the literature, the colistin resistant strain exhibits an “unconventional” drug susceptibility profile, showing increased susceptibility to drugs such as vancomycin or carbapenems. This effect was reminiscent to the previously described “Antibiotic Seesaw Effect”, whereby increased resistance to one class of antibiotics results in increased susceptibility to other classes (Vignaroli, Rinaldi et al. 2011, Vidaillac, Benichou et al. 2012, Ortwine, Werth et al. 2013, Barber, Ireland et al. 2014). Taking advantage of this phenotype, unconventional drug combinations have been tested and successfully developed to treat infections caused by MDR bacteria. The most studied example is infections caused by vancomycin-intermediate resistant MRSA successfully treated by vancomycin and β -lactam combinations (Sieradzki and Tomasz 1997, Vignaroli, Rinaldi et al. 2011, Ortwine, Werth et al. 2013, Barber, Ireland et al. 2014, Dilworth, Ibrahim et al. 2014). A similar phenomenon was suggested in the literature with colistin resistance in Gram-negative bacteria, and strangely enough,

colistin and vancomycin combination was found to be efficient and synergistic against cultures of *P. aeruginosa* and *K. pneumonia* (Vidaillac, Benichou et al. 2012). Unfortunately, there was no data available regarding increased efficacy in the less favorable scenario of biofilms. In this work, it was demonstrated that the combination of vancomycin and colistin is synergistic only against the colistin resistant derivative strain. However, synergy can be achieved only following induced biofilm dispersal supporting the therapeutic potential of this strategy. More conventional combinations against colistin resistant strains should be considered as the difference in susceptibility profile suggests; a combinatorial treatment of colistin and carbapenems for example.

To further gain insights on the mechanism by which the colistin resistant derivative strain becomes more susceptible to unconventional therapy in planktonic cultures and biofilm settings, whole-genome sequencing was performed for the parent PAO1/*p_{BAD}-yhjH* versus its colistin resistant derivative. Dominant point mutations in genes encoding proteins regulating or involved in the biosynthesis of Lipid-A were identified. Amongst them, mutations in *tpbA*, a gene related to the production of eDNA and other matrix related components was found. The two-component system TpbA/B has been found to regulate an antibiotic resistant *P. aeruginosa* SCV phenotype (Valentini and Filloux 2016), as was evident in this work. The *tpbA* mutant phenotype has been shown before to culminate in a high intercellular c-di-GMP content in cells, due to TpbB DGC activity (Pu and Wood 2010). If the colistin resistant evolved strain SCV phenotype is dependent upon high intercellular c-di-GMP equilibrium, induction of the PDE YhjH would have more impact on those tolerance mechanisms.

Other mutations with frequencies of 70-90% were found to be in the locus PA0727 (**Supplementary Table 1**), which is related to the *Pf* filamentous phage in PAO1 (Martínez and Campos-Gómez 2016). This gene is known to be hypermutable and may play a key role in changing the PAO1 EPS constituents, and also promote SCV phenotypes (Kirisits, Prost et al. 2005). These mutations might contribute to the observed differences between colistin susceptible and resistant derivative strains and should be further investigated.

Point mutations in *phoQ* and *lpxC* were identified in the colistin resistant derivative strain, as is commonly observed in literature (Lee, Park et al. 2016). These two genes are involved in the biosynthesis of lipid-A located in the bacterial membranes and the biological target of colistin. These led to a different differential gene expression in the resistant strain: upregulation of the whole *arnBCADTEF-pmrE* operon (Fernández, Gooderham et al. 2010) was evident. Through catalyzed action of this enzymatic cascade transcribed by the operon, the modifications hide the negative charge of Lipid-A and limit binding of polymyxins (Breidenstein, de la Fuente-Núñez et al. 2011, Miller, Brannon et al. 2011). The PhoP/Q and PmrA/B two-component systems, are central signal transduction systems, variations in their expression also were shown to result in changes to other cellular attributes, such as known virulence factor production and motility, leading to a SCV phenotype (Rodrigue, Quentin et al. 2000). It has been suggested that excretion of bacterial Lipid-A increases virulence, as it is a main component of LPS (Maldonado, Sá-Correia et al. 2016).

The product of the plasmid mediated colistin resistance *mcr-1* gene, is a phosphoethanolamine transferase, which was shown in *E. coli* to catalyze the addition of phosphoethanolamine to Lipid-A (Liu, Wang et al. 2016). This plasmid has been since shown to be transmittable to all Gram-negative ESKAPE (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) pathogens which are often treated with colistin (Liu, Chandler et al. 2017). The *mcr-1* enzyme has been shown to increase resistance in *K. pneumoniae* & *A. baumannii* but not in *P. aeruginosa*. *K. pneumoniae* and *P. aeruginosa* both have the *arn* pathway for arabinose addition in Lipid-A and *A. baumannii* has its own phosphoethanolamine transferase, *PmrC* (Adams, Nickel et al. 2009). It is safe to assume that resistance to colistin, inherited or via horizontal gene transfer does constitute a change to Lipid-A and thus changes the properties of the outer membrane. This change will thus facilitate a significant change to the susceptibility profile of resistant strains as a “seesaw effect”.

Once the new potential for antibiotic combinations increases, induced dispersal via depletion of intracellular c-di-GMP, was shown here to allow sufficient penetration and synergy. Future experiments with clinical strains and other bacterial species, such as *K. pneumonia* and *A. baumannii*, are now warranted; to assess the potential of c-di-GMP mediated biofilm dispersal further, to prove that the observed eradication by antimicrobial combinations is not limited to this strain alone. Work was initiated on transforming *K. pneumonia KP1* with the plasmids pJN105 and p_{BAD}-*yhjH*. Evaluation of unconventional treatments and c-di-GMP mediated biofilm dispersal as a

strategy to treat multi-species biofilms of ESKAPE such as *P. aeruginosa* and *S. aureus*.

Experiments described in this work were based on static biofilm models. Even though these models enable high-throughput investigation of drug combinations, there is a need to examine the effects of organisms growing in continuous culture conditions. Ongoing experiments utilizing the DTU flow cell system (data not shown) with the PAO1/*p_{BAD}-yhjH* treated with different antimicrobial combinations following dispersal exhibit a positive trend for biofilm eradication, although further experimentation and optimization are warranted. Additionally, the results shown in this thesis could serve as the basis for future experiments using the CDC biofilm reactor (Larimer, Winder et al. 2016) to study the effect on biofilms when exposed to antimicrobial concentrations mimicking therapeutic regimes.

Finally, following these proof-of-concept studies, c-di-GMP mediated biofilm dispersal should now be tested with potential biofilm dispersal agents. Experimentation with different enzymes that are known to facilitate dispersal, such as DNase and PslG (Yu, Su et al. 2015, Gonzalez Moreno, Trampuz et al. 2017) is ongoing; results are promising, showing similar trends as with the *p_{BAD}-yhjH* construct.

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Appendix

Supplementary Table 1 SNP between parent and colistin resistant PAO1/P_{BAD⁻}*yhjH* by pairwise comparison of whole genome sequencing.

Gene ID	Region	Type	Reference	Allele	Amino acid change	Frequency (%)	Product
phoQ	1279684	SNV	G	T	NP_249871.1.p.Glu435*	100.00	two-component sensor PhoQ
tpbA	4351381	SNV	C	G	NP_252574.1.p.Ala183Gly	100.00	protein tyrosine phosphatase TpbA
tpbA	4351393	SNV	G	A	NP_252574.1.p.Gly187Asp	100.00	protein tyrosine phosphatase TpbA
tpbA	4351395	SNV	C	A	NP_252574.1.p.Leu188Met	100.00	protein tyrosine phosphatase TpbA
tpbA	4351387..4351388	MNV	TC	AG	NP_252574.1.p.Val185Glu	100.00	protein tyrosine phosphatase TpbA
lpxC	4938732	SNV	G	T	NP_253096.1.p.Phe152Leu	100.00	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase
PA0727	795031	SNV	C	T		94.35	hypothetical protein
PA0727	795043	SNV	T	C		94.34	hypothetical protein
PA0727	795040	SNV	T	C		94.33	hypothetical protein
PA0727	795028	SNV	G	T		94.20	hypothetical protein
PA0727	794974	SNV	C	A		94.04	hypothetical protein
PA0727	795370	SNV	T	C		94.01	hypothetical protein
PA0727	794995	SNV	C	T		94.01	hypothetical protein
PA0727	794977	SNV	T	C		93.88	hypothetical protein
PA0727	795025	SNV	G	C		93.54	hypothetical protein
PA0727	794989..794990	MNV	CT	GC		93.31	hypothetical protein
PA0727	795385	SNV	T	C		91.74	hypothetical protein
PA0727	795391	SNV	G	C		91.16	hypothetical protein
PA0727	795019^795020	Insertion	-	T	NP_249418.1.p.Asp174fs	90.72	hypothetical protein
PA0727	795009..795010	MNV	AA	CG	NP_249418.1.p.Lys170Thr	90.47	hypothetical protein
PA0727	795013	SNV	G	T		90.32	hypothetical protein
PA0727	795016..795017	Replacement	TC	G	NP_249418.1.p.Arg173fs	89.74	hypothetical protein
PA0727	795403	SNV	C	T		88.73	hypothetical protein
PA0727	795406	SNV	G	A		87.64	hypothetical protein
PA0727	795409	SNV	A	G		85.80	hypothetical protein
PA0727	795412	SNV	A	G		84.48	hypothetical protein
PA0727	795424	SNV	C	T		73.52	hypothetical protein

Supplementary Table 2 Differentially upregulated genes in the colistin resistant PAO1/P_{BAD}-*yhjH* strain relative to its parent strain.

Gene ID	log2FoldChange	PseudoCAP Function Class
PA3559	8.271	Putative enzymes
arnA	8.206	Putative enzymes; Adaptation, Protection; Antibiotic resistance and susceptibility; Cell wall / LPS / capsule
arnC	8.173	Adaptation, Protection; Putative enzymes; Cell wall / LPS / capsule; Antibiotic resistance and susceptibility
arnB	8.094	Cell wall / LPS / capsule; Antibiotic resistance and susceptibility; Adaptation, Protection
arnD	7.735	Adaptation, Protection; Antibiotic resistance and susceptibility; Cell wall / LPS / capsule
arnT	7.324	Adaptation, Protection; Membrane proteins; Cell wall / LPS / capsule
arnE	6.747	Adaptation, Protection; Cell wall / LPS / capsule; Membrane proteins
arnF	6.591	Membrane proteins; Adaptation, Protection; Cell wall / LPS / capsule
oprH	6.242	Membrane proteins; Adaptation, Protection; Transport of small molecules
phoP	5.665	Transcriptional regulators; Two-component regulatory systems
phoQ	5.264	Two-component regulatory systems
PA4517	4.654	Hypothetical, unclassified, unknown
tpbA	4.652	Motility & Attachment; Translation, post-translational modification, degradation
PA4011	4.191	Membrane proteins
gcvP2	4.071	Central intermediary metabolism; Amino acid biosynthesis and metabolism
dctA	3.944	Transport of small molecules
PA4010	3.893	Hypothetical, unclassified, unknown
gcvH2	3.467	Amino acid biosynthesis and metabolism
PA1181	2.870	Membrane proteins
inaA	2.716	Adaptation, Protection
glyA2	2.613	Amino acid biosynthesis and metabolism
PA4379	2.606	Hypothetical, unclassified, unknown
PA5168	2.557	Membrane proteins; Transport of small molecules
dkgA	2.515	Fatty acid and phospholipid metabolism
PA5167	2.448	Membrane proteins; Transport of small molecules
PA0921	2.397	Membrane proteins
PA5169	2.309	Membrane proteins; Transport of small molecules
PA1053	2.271	Membrane proteins
PA4635	2.239	Hypothetical, unclassified, unknown
PA2112	2.212	Hypothetical, unclassified, unknown
PA1972	2.105	Membrane proteins
PA1344	2.087	Putative enzymes
PA4455	2.001	Membrane proteins; Transport of small molecules
PA2110	1.992	Hypothetical, unclassified, unknown
PA2111	1.968	Hypothetical, unclassified, unknown
PA4824	1.966	Hypothetical, unclassified, unknown
PA0920	1.961	Membrane proteins
PA4586	1.921	Membrane proteins
opdO	1.918	Transport of small molecules; Membrane proteins
PA2173	1.852	Hypothetical, unclassified, unknown
PA0924	1.841	Hypothetical, unclassified, unknown; Membrane proteins
liuA	1.841	Carbon compound catabolism
mgtA	1.822	Transport of small molecules
PA3310	1.807	Membrane proteins
PA4456	1.799	Transport of small molecules
PA4773	1.787	Hypothetical, unclassified, unknown
liuR	1.739	Transcriptional regulators
pcoA	1.732	Adaptation, Protection
PA0320	1.708	Hypothetical, unclassified, unknown
pcoB	1.650	Adaptation, Protection
PA2159	1.627	Hypothetical, unclassified, unknown
PA1592	1.619	Hypothetical, unclassified, unknown
aruG	1.613	Amino acid biosynthesis and metabolism
PA2116	1.597	Hypothetical, unclassified, unknown
PA5530	1.578	Membrane proteins; Transport of small molecules
PA4454	1.569	Hypothetical, unclassified, unknown
PA4452	1.560	Hypothetical, unclassified, unknown
PA2114	1.554	Membrane proteins; Transport of small molecules
psiN	1.541	Hypothetical, unclassified, unknown
glpD	1.540	Central intermediary metabolism; Energy metabolism
PA4596	1.536	Transcriptional regulators
PA3274	1.515	Hypothetical, unclassified, unknown
PA0049	1.513	Hypothetical, unclassified, unknown
PA4453	1.503	Hypothetical, unclassified, unknown
sdaA	1.502	Amino acid biosynthesis and metabolism
PA4822	1.484	Membrane proteins
PA2109	1.474	Hypothetical, unclassified, unknown
PA2457	1.472	Hypothetical, unclassified, unknown
PA1323	1.467	Hypothetical, unclassified, unknown
PA4382	1.440	Hypothetical, unclassified, unknown
PA1688	1.439	Hypothetical, unclassified, unknown
liuB	1.437	Carbon compound catabolism
PA3523	1.431	Transport of small molecules

PA4774	1.428	Hypothetical, unclassified, unknown
glpK	1.428	Central intermediary metabolism
PA2557	1.409	Fatty acid and phospholipid metabolism
PA1471	1.407	Hypothetical, unclassified, unknown
rfaD	1.400	Cell wall / LPS / capsule
PA4377	1.398	Hypothetical, unclassified, unknown
aruF	1.391	Amino acid biosynthesis and metabolism
PA3691	1.391	Hypothetical, unclassified, unknown
PA5526	1.382	Hypothetical, unclassified, unknown
PA2167	1.377	Hypothetical, unclassified, unknown
PA1404	1.356	Hypothetical, unclassified, unknown
pfl	1.356	Translation, post-translational modification, degradation
plcR	1.355	Secreted Factors (toxins, enzymes, alginate)
PA5212	1.351	Hypothetical, unclassified, unknown
opmD	1.350	Membrane proteins; Transport of small molecules
PA4826	1.346	Hypothetical, unclassified, unknown
PA1345	1.338	Hypothetical, unclassified, unknown
PA0833	1.335	Membrane proteins
liuC	1.324	Carbon compound catabolism
PA0919	1.320	Hypothetical, unclassified, unknown
PA3819	1.318	Membrane proteins
osmE	1.315	Membrane proteins; Adaptation, Protection
PA2486	1.311	Hypothetical, unclassified, unknown
PA4023	1.310	Transport of small molecules
polA	1.309	DNA replication, recombination, modification and repair
PA2171	1.288	Hypothetical, unclassified, unknown
PA4877	1.283	Hypothetical, unclassified, unknown
PA0737	1.277	Hypothetical, unclassified, unknown
PA1689	1.271	Hypothetical, unclassified, unknown
PA5183	1.261	Membrane proteins
PA4172	1.261	DNA replication, recombination, modification and repair
PA2164	1.255	Putative enzymes
PA2168	1.254	Hypothetical, unclassified, unknown
fecA	1.254	Membrane proteins; Transport of small molecules
PA4577	1.249	Hypothetical, unclassified, unknown
gcvT2	1.232	Central intermediary metabolism; Amino acid biosynthesis and metabolism
PA1196	1.225	Transcriptional regulators
PA5460	1.225	Hypothetical, unclassified, unknown
PA2143	1.216	Hypothetical, unclassified, unknown
PA2807	1.209	Hypothetical, unclassified, unknown
liuD	1.202	Carbon compound catabolism
osmC	1.199	Adaptation, Protection
mexI	1.193	Membrane proteins; Transport of small molecules
treA	1.192	Carbon compound catabolism
PA4828	1.189	Hypothetical, unclassified, unknown
PA0490	1.184	Hypothetical, unclassified, unknown
PA1324	1.178	Hypothetical, unclassified, unknown
PA1494	1.164	Hypothetical, unclassified, unknown
ampDh3	1.154	Antibiotic resistance and susceptibility
PA2562	1.151	Hypothetical, unclassified, unknown
aruD	1.150	Amino acid biosynthesis and metabolism
PA2190	1.147	Hypothetical, unclassified, unknown
PA3519	1.146	Hypothetical, unclassified, unknown
PA2163	1.138	Hypothetical, unclassified, unknown
PA0462	1.136	Hypothetical, unclassified, unknown
PA4357	1.136	Hypothetical, unclassified, unknown
PA2046	1.134	Hypothetical, unclassified, unknown
arcD	1.132	Membrane proteins; Amino acid biosynthesis and metabolism; Transport of small molecules
PA4359	1.131	Hypothetical, unclassified, unknown
PA3839	1.128	Membrane proteins; Transport of small molecules
psrA	1.122	Transcriptional regulators
PA2414	1.121	Carbon compound catabolism
PA2415	1.120	Membrane proteins
PA2169	1.118	Hypothetical, unclassified, unknown
PA2021	1.104	Hypothetical, unclassified, unknown
PA2655	1.098	Hypothetical, unclassified, unknown
PA4610	1.096	Hypothetical, unclassified, unknown
mucA	1.095	Transcriptional regulators; Cell wall / LPS / capsule
lptF	1.094	Membrane proteins; Transport of small molecules
PA0028	1.093	Hypothetical, unclassified, unknown
PA5027	1.082	Hypothetical, unclassified, unknown
PA1111	1.072	Hypothetical, unclassified, unknown
mexH	1.069	Transport of small molecules
PA3459	1.068	Amino acid biosynthesis and metabolism

glcF	1.066	Central intermediary metabolism; Carbon compound catabolism
PA3278	1.058	Membrane proteins
PA2160	1.056	Putative enzymes
PA0062	1.051	Hypothetical, unclassified, unknown
phzH	1.045	Putative enzymes
PA4878	1.043	Transcriptional regulators
PA3922	1.037	Hypothetical, unclassified, unknown
PA3460	1.035	Putative enzymes
PA2134	1.028	Hypothetical, unclassified, unknown
PA2753	1.026	Hypothetical, unclassified, unknown
PA1282	1.024	Membrane proteins; Transport of small molecules
PA4358	1.019	Membrane proteins; Transport of small molecules
glpF	1.018	Transport of small molecules
PA4338	1.014	Membrane proteins
PA5182	1.013	Membrane proteins
PA3880	1.010	Hypothetical, unclassified, unknown
phuR	1.007	Transport of small molecules
PA0460	1.004	Hypothetical, unclassified, unknown

Supplementary Table 3 Differentially downregulated genes in the colistin resistant PAO1/*P_{BAD}-yhjH* strain relative to its parent strain.

Gene ID	log2FoldChange	PseudoCAP Function Class
PA3441	-3.637	Transport of small molecules
PA3443	-2.900	Membrane proteins; Transport of small molecules
PA3442	-2.758	Transport of small molecules
PA2309	-2.365	Hypothetical, unclassified, unknown
PA5181	-2.304	Putative enzymes
tauD	-2.300	Carbon compound catabolism
PA3445	-2.253	Hypothetical, unclassified, unknown
PA2840	-2.212	Transcription, RNA processing and degradation
PA2086	-2.178	Carbon compound catabolism
PA5099	-2.171	Membrane proteins; Transport of small molecules
hutU	-2.151	Amino acid biosynthesis and metabolism
hutH	-2.140	Amino acid biosynthesis and metabolism
PA2310	-2.044	Putative enzymes
PA2308	-2.017	Transport of small molecules
PA2307	-2.014	Membrane proteins; Transport of small molecules
rpmB	-1.977	Translation, post-translational modification, degradation
PA5470	-1.934	Translation, post-translational modification, degradation
PA3931	-1.932	Hypothetical, unclassified, unknown
PA5097	-1.907	Membrane proteins; Transport of small molecules
PA3447	-1.846	Transport of small molecules
PA3446	-1.782	Hypothetical, unclassified, unknown
PA2085	-1.765	Carbon compound catabolism
PA3741	-1.758	Hypothetical, unclassified, unknown
PA3932	-1.736	Transcriptional regulators
rpmG	-1.734	Translation, post-translational modification, degradation
PA3938	-1.706	Transport of small molecules
PA5471	-1.660	Antibiotic resistance and susceptibility
PA3936	-1.623	Membrane proteins; Transport of small molecules
PA3450	-1.611	Adaptation, Protection
PA2050	-1.597	Transcriptional regulators
PA4191	-1.566	Putative enzymes
mdcD	-1.556	Carbon compound catabolism
PA0578	-1.552	Hypothetical, unclassified, unknown
PA2090	-1.530	Hypothetical, unclassified, unknown
PA5106	-1.499	Hypothetical, unclassified, unknown
rpsT	-1.486	Central intermediary metabolism; Translation, post-translational modification, degradation
atsA	-1.473	Central intermediary metabolism; Carbon compound catabolism
rplS	-1.464	Translation, post-translational modification, degradation
PA4918	-1.459	Hypothetical, unclassified, unknown
PA4195	-1.457	Transport of small molecules
PA4881	-1.451	Hypothetical, unclassified, unknown
PA4933	-1.441	Membrane proteins
PA2312	-1.435	Transcriptional regulators
PA5072	-1.429	Adaptation, Protection; Chemotaxis
PA5096	-1.414	Transport of small molecules
PA4582	-1.406	Hypothetical, unclassified, unknown
PA5383	-1.392	Hypothetical, unclassified, unknown
trmD	-1.383	Transcription, RNA processing and degradation
PA5180	-1.377	Hypothetical, unclassified, unknown
PA4355	-1.362	Membrane proteins; Transport of small molecules; Transcriptional regulators
pscB	-1.354	Protein secretion/export apparatus
ibpA	-1.342	Chaperones & heat shock proteins
PA3937	-1.329	Transport of small molecules
PA5103	-1.324	Hypothetical, unclassified, unknown
PA2597	-1.309	Putative enzymes
PA5504	-1.287	Membrane proteins; Transport of small molecules
PA0191	-1.286	Transcriptional regulators
PA2328	-1.286	Hypothetical, unclassified, unknown
PA3444	-1.283	Putative enzymes
PA2600	-1.274	Hypothetical, unclassified, unknown
PA2019	-1.259	Transport of small molecules; Antibiotic resistance and susceptibility
PA2326	-1.252	Putative enzymes
PA0805	-1.246	Hypothetical, unclassified, unknown
PA2083	-1.246	Carbon compound catabolism
hutG	-1.235	Amino acid biosynthesis and metabolism
PA2594	-1.230	Hypothetical, unclassified, unknown
hsiU	-1.216	Chaperones & heat shock proteins
PA2204	-1.215	Transport of small molecules
PA2334	-1.210	Transcriptional regulators
grpE	-1.210	DNA replication, recombination, modification and repair; Chaperones & heat shock proteins
PA5095	-1.203	Membrane proteins; Transport of small molecules
rpsR	-1.201	Translation, post-translational modification, degradation
hutI	-1.199	Amino acid biosynthesis and metabolism

rplJ	-1.195	Translation, post-translational modification, degradation
PA5503	-1.189	Transport of small molecules
PA2018	-1.176	Transport of small molecules; Membrane proteins; Antibiotic resistance and susceptibility
PA2540	-1.175	Hypothetical, unclassified, unknown
PA4220	-1.172	Hypothetical, unclassified, unknown
pscD	-1.166	Protein secretion/export apparatus
rplI	-1.163	Translation, post-translational modification, degradation
PA2971	-1.162	Hypothetical, unclassified, unknown
glbB	-1.161	Amino acid biosynthesis and metabolism
PA2359	-1.157	Transcriptional regulators
mreD	-1.157	Cell wall / LPS / capsule; Cell division
PA1501	-1.156	Hypothetical, unclassified, unknown
pncB1	-1.156	Biosynthesis of cofactors, prosthetic groups and carriers
PA3600	-1.153	Translation, post-translational modification, degradation
rpsF	-1.137	Translation, post-translational modification, degradation
PA0434	-1.134	Membrane proteins
PA5094	-1.129	Transport of small molecules
PA0277	-1.128	Hypothetical, unclassified, unknown
PA2598	-1.124	Hypothetical, unclassified, unknown
arsB	-1.118	Membrane proteins; Transport of small molecules
PA1791	-1.116	Hypothetical, unclassified, unknown
dnaK	-1.111	DNA replication, recombination, modification and repair; Adaptation, Protection; Chaperones & heat shock proteins
PA2084	-1.111	Amino acid biosynthesis and metabolism
flhA	-1.101	Chemotaxis; Adaptation, Protection; Motility & Attachment
mdcE	-1.096	Carbon compound catabolism
aprE	-1.094	Secreted Factors (toxins, enzymes, alginate); Protein secretion/export apparatus
PA2786	-1.092	Hypothetical, unclassified, unknown
rplU	-1.089	Translation, post-translational modification, degradation
PA0879	-1.088	Putative enzymes
thiI	-1.087	Biosynthesis of cofactors, prosthetic groups and carriers
clpB	-1.086	Translation, post-translational modification, degradation
PA5090	-1.085	Hypothetical, unclassified, unknown
rbfA	-1.084	Translation, post-translational modification, degradation; Adaptation, Protection
hutC	-1.069	Transcriptional regulators
PA1697	-1.062	Protein secretion/export apparatus
PA3939	-1.059	Hypothetical, unclassified, unknown
rplL	-1.056	Translation, post-translational modification, degradation
arsR	-1.051	Transcriptional regulators
aprF	-1.026	Secreted Factors (toxins, enzymes, alginate); Protein secretion/export apparatus
rpmA	-1.019	Translation, post-translational modification, degradation
PA2327	-1.019	Membrane proteins; Transport of small molecules
pscC	-1.005	Protein secretion/export apparatus