universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Synthesis of the RNA CrcZ during anaerobiosis and impact of the CrcZ levels on anoxic biofilm formation and antibiotic susceptibility of *Pseudomonas aeruginosa*"

verfasst von / submitted by

Rim Chriki

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna, 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 877
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Genetik und Entwicklungsbiologie
Betreut von / Supervisor:	Univ.-Prof. Dr. Udo Bläsi

Acknowledgments

First and foremost, I would like to express my deepest appreciation to my supervisor Prof. Dr. Udo Bläsi for accepting me in his research group and for his continuous support, guidance and advice during this work.

I would also like to extend my deepest gratitude to Dr. Petra Pusic for her support, help and encouragement. Thank you for always finding time to answer my incessant questions.

I also thank all lab members for their help and for the nice time that we spent together, particularly Dr. Elisabeth Sonnleitner and Msc Beatrice Krennmayer.

Special thanks are owed to my parents, Ali and Saloua, for their endless support, encouragement, love and unwavering belief in me. I thank my brother, Mohamed Ali, for his emotional support and kind words. I also thank my husband, Adeeb, who has provided moral support.

A final thank goes to my children, Sana and Nabil, for their smiles, laughter and endless love. Thank you for making my life so beautiful.

Table of Contents

1. Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod shaped bacterium (Figure 1) belonging to the *Gammaproteobacteria*. *P. aeruginosa* cells are 3-4 µm long and 0.5-1.0 µm wide, and most of the cells possess a single polar flagellum (Krieg & Holt, 1984). *P. aeruginosa* produces the pigment pyocyanin when grown on minimal medium or during infections, giving it a greenish-blue appearance (Govan & Deretic, 1996).

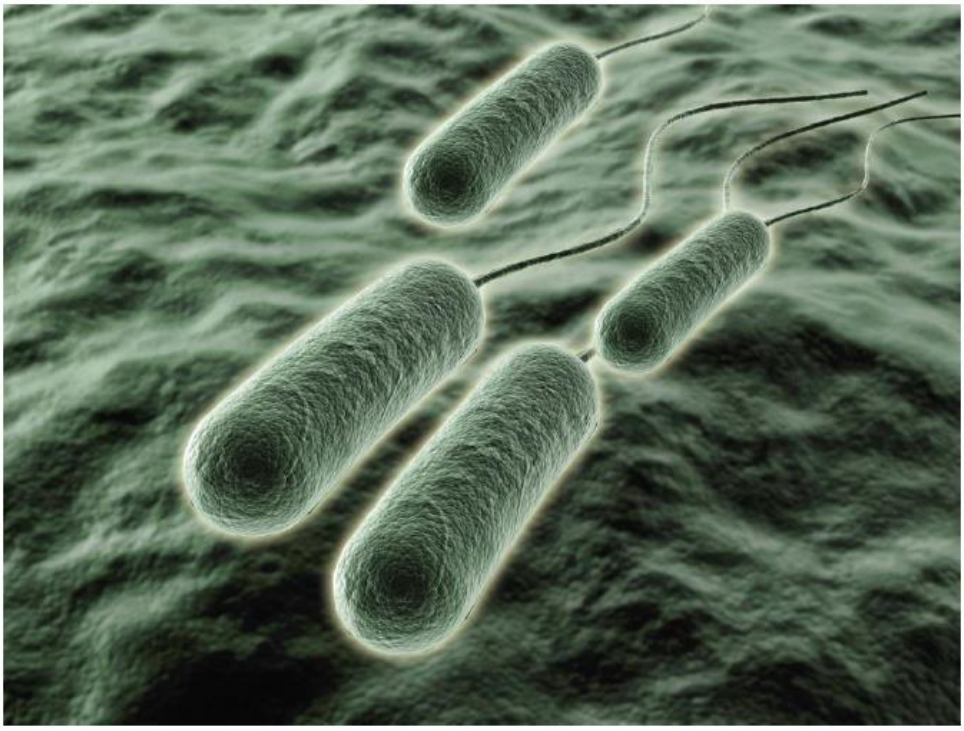

Figure 1. Scanning electron micrograph (SEM) of *P. aeruginosa*. Figure taken from Sandle (2014).

The size of the complete *P. aeruginosa* PAO1 genome is about 6.3 Mbp (Stover *et al.*, 2000), encoding ~ 5,770 open reading frames. *P. aeruginosa* is known to be versatile in its ability to degrade a wide variety of different organic molecules to be used as carbon and energy sources, which enables it to inhabit a wide range of ecological niches (Ornston, 1971). *P. aeruginosa* is found ubiquitously in water, air, soil, animals, and plants (Kucera & Lysenko, 1968; Kominos *et al*., 1972; Mahajan-Miklos *et al*., 1999). This versatility is believed to result from its relatively large genome size and the complicated cellular regulatory network, as indicated by the presence of more than 90 two-component regulatory systems (Stover *et al.*, 2000).

P. aeruginosa is an opportunistic human pathogen with a remarkable capacity to cause diseases in susceptible hosts. It is the major microbial pathogen of cystic fibrosis (CF) patients. Besides causing chronic CF lung infections, *P. aeruginosa* has been found to cause a variety of acute infections, including common acute septicemia from burn or surgical wounds, urinary tract infections, corneal ulceration, endocarditis and pneumonia in immunocompromised patients (Van Delden & Iglewski, 1998; Tatterson *et al*., 2001; Kerr & Snelling, 2009; Valderrey *et al.*, 2010).

P. aeruginosa comprises both, cell-associated and extracellular virulence factors. The cell-associated factors include lipopolysaccharide, flagella and type IV pili, and the exopolysaccharide alginate. The secreted factors are toxins, elastase and other proteases, phospholipases and small molecules such as phenazines, rhamnolipids and cyanide (Van Delden & Iglewski, 1998; Sonnleitner *et al*., 2003; Yahr & Wolfgang, 2006; Gooderham & Hancock, 2008).

The production of cell-to-cell connecting extracellular polymeric substances (EPS) are required for the formation of biofilms (Donlan, 2002; Pamp *et al*., 2007; Karatan & Watnick, 2009; Tolker-Nielsen, 2015 ; Ciofu & Tolker-Nielsen, 2019). EPS is composed mainly of exopolysaccharides, extracellular DNA (eDNA), and polypeptides that form a highly hydrated polar mixture that contributes to the overall structural scaffold and architecture of the biofilm (Sutherland, 2001; Barken *et al*., 2008; Flemming & Wingender, 2010; Rasamiravaka *et al*., 2015; Tolker-Nielsen, 2015 ; Ciofu & Tolker-Nielsen, 2019). In addition, the outer membrane proteins and a variety of cell appendages such as cup fimbriae, type IV pili and flagella are also involved in connecting bacteria-to-bacteria and can therefore be considered to be part of the biofilm matrix (Ciofu *et al*., 2015). Although the presence of a cell-to-cell connecting matrix appears to be a common feature of microbial biofilms, there is a remarkable diversity in the composition of these matrices. It is anticipated that the capacity of bacteria to produce different biofilm matrix components allows colonization of different niches through different biofilm formation pathways (Pamp *et al*., 2007; Ciofu *et al*., 2015). In cystic fibrosis patients, *P. areruginosa* forms clusters of cells in the airway mucus that are surrounded by inflammatory polynuclear cells consuming a large proportion of the available oxygen. In this case, the formed anoxic biofilms are not surface attached (Worlitzsch *et al*., 2002; Kolpen *et al*., 2010; Aanaes *et al*., 2011; Bjarnsholt *et al*., 2013; Jensen *et al*., 2017). On the contrary, in nosocomial ventilator-associated pneumonia, the biofilms are attached to endotracheal tubes, and are most likely localized in an aerobic environment. Thus, the bacteria in the periphery of these biofilms experience aerobic conditions (Høiby *et al*., 2015).

The most notable feature of biofilms is their recalcitrance to antibiotic treatments (Costerton *et al*., 1999; Kirisits & Parsek, 2006; Lewis, 2010; Liao & Sauer, 2012; Ciofu *et al*., 2015; Ciofu & Tolker-Nielsen, 2019). Biofilms persist and are hard to eradicate because of mechanisms that involve restricted penetration of antimicrobials, differential physiological activity, efflux systems, and enhanced repair systems (Brown & Smith, 2003; Mah *et al*.,

2003; Walters *et al*., 2003; Werner *et al*., 2004; Pamp *et al*., 2008; Zhang & Mah, 2008; De Groote *et al*. 2009; Lewis, 2010; Alhede *et al*., 2011; Liao & Sauer, 2012; Zhang *et al*., 2012; Ciofu *et al*., 2015; Ciofu *et al*., 2017). These tolerance mechanisms not only lead to the emergence of persisters, but also of antibiotic-resistant mutants (Ciofu & Tolker-Nielsen, 2019). Both resistance and tolerance are involved in the recalcitrance of biofilms to antibiotic treatment (Lebeaux *et al*., 2014).

Over the past decades, antibiotic resistant bacteria are increasing due to the misuse and abuse of antibiotics that are known to create selective pressure, which in turn provides a competitive advantage for antibiotic resistant mutants. In addition to increased resistance to existing antibiotics, there is a lack of development and introduction of new antimicrobials. Thus, bacterial resistance to all classes of antibiotics is becoming a serious threat to human health (Fernández & Hancock, 2012; Laxminarayan *et al.*, 2013; Fair & Tor, 2014; Rasamiravaka *et al*., 2015; Blair *et al*., 2015).

There are three principal types of antibiotic resistance; namely, intrinsic, acquired, and adaptive (Fernández & Hancock, 2012). Intrinsic resistance comprises all the inherent structural or functional characteristics of a particular bacterial species that enables it to resist the action of a certain antibiotic. An example for intrinsic resistance is the absence of a susceptible target of a specific antibiotic. In addition to intrinsic resistance, bacteria can acquire or develop resistance to antibiotics either by incorporating new genetic material (plasmids, transposons, integrons, and naked DNA, etc.) or as a result of mutations (Fernández & Hanock, 2012; Blair *et al*., 2015). There are three main groups of mechanisms that mediate acquired resistance: first, those that minimize the intracellular concentrations of the antibiotic as a result of poor penetration into the bacterium or of antibiotic efflux; second, those that modify the antibiotic target by genetic mutation or post-translational modification of the target; and third, those that inactivate the antibiotic by hydrolysis or modification (Blair *et al*., 2015). Both intrinsic and acquired resistance are characterized by an irreversible phenotype and are independent of the presence of the antibiotic or of environmental conditions (Fernández *et al*., 2011). In contrast, adaptive antibiotic resistance can be developed as a result of an environmental stimulus such as sub-inhibitory levels of antimicrobials, pH, anaerobiosis, cation levels or nutrient cues, etc., as well as social activities like biofilm formation and swarming motility leading to temporary adaptations in gene expression levels, which are typically lost after removal of the trigger; although, in many cases the original level of resistance cannot be restored (Mawer & Greenwood, 1978; Gilleland *et al.*, 1989; Karlowsky *et al.*, 1997; Poole, 2005; Fernández *et al.*, 2011; Skiada *et al.*, 2011; Fernández & Hancock, 2012). Whereas intrinsic and aquired resistance elements are passed on vertically through bacterial reproduction, adaptive resistance is not inherited due to its transient nature. Moreover, acquired resistance can be transferred horizontally among different bacterial species through plasmids carrying resistance genes, thus increasing the dissemination of antibiotic resistance genes (Table 1) (Blair *et al.*, 2015; Garneau-Tsodikova & Labby, 2016).

Table 1. Comparison between the three major types of antibiotic resistance (Fernández *et al*., 2011).

Type of resistance	Intrinsic	Acquired	Adaptive
Acquisition	Not acquired, part of the genetic landscape of the respective strain or species	Mutation Horizontal gene transfer	Changes in gene expression; triggered by environmental factors or presence of antimicrobials.
Characteristics	Inheritable Stable Irreversible Independent of environmental cues	Inheritable Stable Irreversible Independent of environmental cues	Not inheritable Transient Generally, reverts upon removal of inducing signal Dependent on environmental cues

Microbial biofilms are capable to tolerate antibiotics and components of the host immune system, which complicates the treatment of the infections (Høiby *et al*., 2015). In fact, the current antibiotics cannot completely eradicate the bacteria in biofilms, often leading to a relapse of the infection (Fernandez-Barat *et al*., 2017). Moreover, the high concentrations of antibiotics required for treating biofilm infections (up to 1,000 times higher than for planktonic cells (Macia *et al*., 2014) are toxic *in vivo* if administrated intravenously to a patient (Hengzhuang *et al*., 2012). Therefore, the development of new strategies to treat microbial biofilms is urgent. Several strategies have been employed or proposed to overcome the antibiotic tolerance of biofilms: providing high antibiotic concentrations through topical administration, combined antimicrobials and sequential therapies or the use of adjuvants to improve the efficacy of antibiotics (Høiby *et al*., 2015; Ciofu *et al*., 2017). Moreover, it has been proposed to revive persister cells in order to restore their antibiotic susceptibility. For instance, mannitol was demonstrated to increase the susceptibility of *P. aeruginosa* biofilm persister cells toward the aminoglycoside tobramycin by reverting the persister phenotype through an active physiological response of the bacteria (Barraud *et al*., 2013; Ciofu & Tolker-Nielsen, 2019).

1.2 Small non-coding RNAs in Bacteria

Around 40 years ago, the plasmid- and transposon-encoded antisense small RNAs (sRNAs) RNAI and CopA were found to negatively regulate plasmid copy number in *Escherichia coli* (Stougaard *et al*. 1981; Tomizawa *et al*., 1981). Although, the first detected regulatory sRNAs were discovered on extra-chromosomal genetic elements such as plasmids, transposons, and bacteriophages (Wagner & Simons, 1994), chromosomally encoded sRNAs were identified more recently (Guillier *et al.*, 2006). The bacterial sRNAs constitute a structurally diverse class of molecules that range in size from 70 to 500 nucleotides (nt). These sRNAs

can modulate gene expression by acting as regulators of (i) transcription, (ii) RNA modifications or stability, and (iii) mRNA translation. Most characterized sRNAs regulate gene expression by base-pairing with mRNAs (Storz *et al.*, 2011). These base-pairing sRNAs can be grouped into two general classes: the *cis*-encoded sRNAs, also termed antisense RNAs (asRNAs), are encoded at the same genetic location, but on the opposite strand of the RNA targets, whereas the *trans*-encoded sRNAs are encoded at a chromosomal location distinct from their mRNA targets (Storz *et al*., 2005; Waters & Storz, 2009). While *cis*-encoded sRNAs are complementary to their target, *trans*-encoded sRNAs pair to their target with limited complementarity, often requiring the RNA chaperone Hfq for base-pairing (Valentin-Hansen *et al*., 2004; Aiba, 2007; Brennan & Link, 2007).

1.3. The RNA chaperone Hfq

The RNA chaperone Hfq was discovered and characterized more than 40 years ago in *Escherichia coli* as a host factor required for replication of the bacteriophage Qβ (de Fernandez *et al*., 1968). Hfq belongs to the class of Sm and Sm-like proteins which in Eukaryotes play an important role in nuclear pre-mRNA splicing (Moller *et al*., 2002; Schumacher *et al*., 2002; Zhang *et al*., 2002). The Hfq protein is conserved among bacteria (Sonnleitner *et al.,* 2002). The conserved core of the Hfq promoter is composed of an N-terminal α-helix followed by five β-strands that form a bent sheet that displays the topology β5α1β1β2β3β4. The SM1 motif consists of the first three β-strands (β1-3), whereas the SM2 motif encompasses the β strands 4 and 5 (Figure 2a) (Sauter *et al.*, 2003; Nikulin *et al*., 2005; Brennan & Link, 2007; Vogel & Luisi, 2011). Six of these protomers (each 8-11 kDa) (Figure 2b) form the symmetric Hfq homo-hexamer (Figure 2c). The ring-like architecture of Hfq exposes two faces for potential interaction with nucleic acids. The surface on which the amino-terminal α-helix is exposed is referred to as 'the proximal side', whereas the opposite site is termed 'distal side' (Figure 2d) (Brennan & Link, 2007). The proximal surface preferably binds to uridine-rich stretches often present in sRNAs, whereas the distal surface binds to A-rich regions frequently present in mRNAs (Mikulecky *et al*., 2004; Link *et al*., 2009). Thus, Hfq is able to bring these distinct RNA species into close proximity as part of a sRNA-Hfq-mRNA ternary complex.

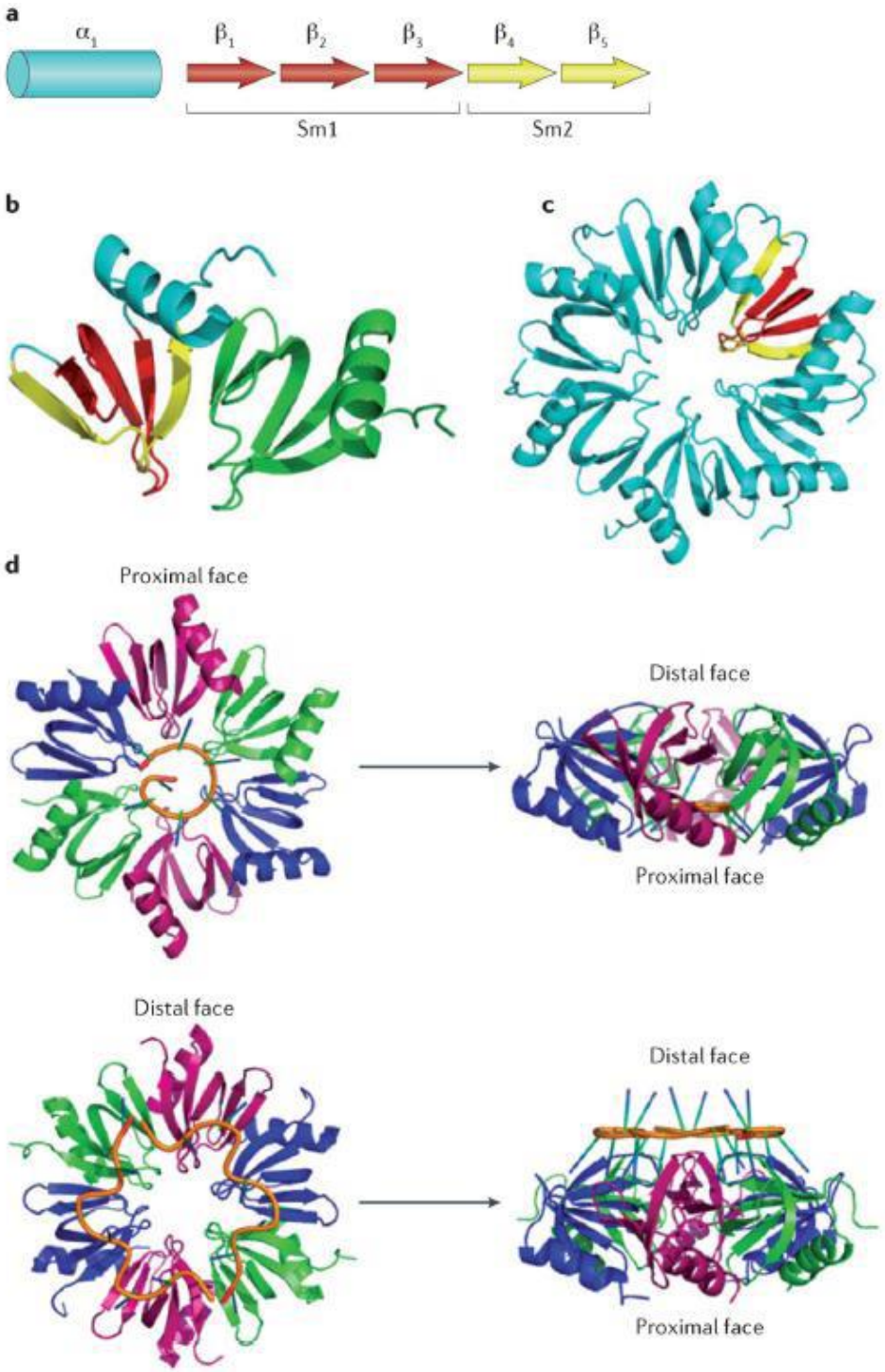

Figure 2. The structure of Hfq and its interaction with RNA. (a) Secondary-structural elements of the Hfq protomer, that consists of a N-terminal α-helix followed by five β-sheets (b) Each protomer is a compact structural unit in which the β-strands form a set of anti-parallel sheets. (c) Six of these protomers form the Hfq hexamer. (d) The proximal RNA binding surface of Hfq (top) and the distal RNA binding surface of Hfq (bottom) are indicated. Bound RNAs are presented in orange. Figure taken from Vogel & Luisi (2011).

Hfq can act as a post-transcriptional regulator of gene expression at the level of translation and/or RNA stability through several well characterized mechanisms (Valentin-Hansen *et al*., 2004; Fröhlich & Vogel, 2009; Vogel & Luisi, 2011; Stanek *et al*., 2017). First, Hfq can assist a cognate sRNA to base-pair with the 5'UTR of its target mRNA, thus, rendering the 5' region inaccessible for ribosomes (Figure 3a). In opposite, Hfq can also mediate the interaction of a sRNA with its target mRNA in order to disrupt a secondary structure that otherwise masks the ribosome binding site (RBS) and inhibits translation (Figure 3b). Furthermore, Hfq can protect sRNAs from endonucleolytic attack thus increasing their stability (Figure 3c) (Moll *et al.*, 2003) or promote endonuclease-mediated cleavage of sRNA-bound mRNAs (Figure 3d) (Aiba, 2007). Finally, Hfq can induce RNA turnover by rendering the 3' ends accessible for polyadenylation, which in turn triggers 3'-to-5' exonucleolytic degradation (Figure 3e) (Vogel & Luisi, 2011).

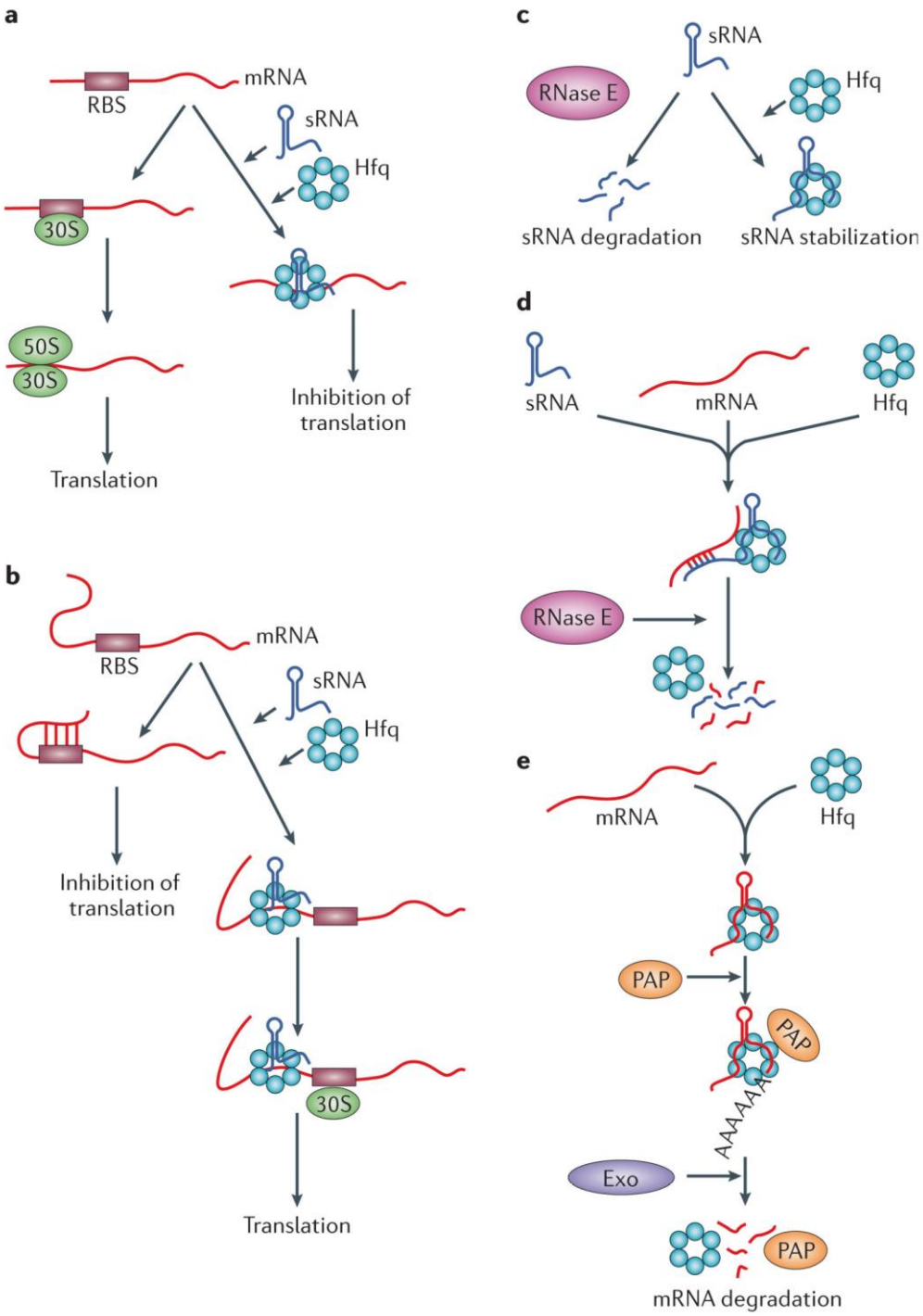

Figure 3. Post-transcriptional regulation mechanisms of Hfq. (a) Hfq in association with a sRNA can prevent ribosome binding, thus repressing the translation of a given mRNA. **(b)** A complex formed by Hfq and a specific sRNA can disrupt the secondary structure of a target mRNA that otherwise masks the RBS and inhibits translation. **(c)** Hfq may protect some sRNAs from cleavage by ribonucleases such as ribonuclease E (RNaseE). **(d)** Hfq may promote endonuclease-mediated cleavage of certain sRNAs and their target mRNAs. **(e)** Hfq may induce polyadenylation by poly(A) polymerase (PAP) of a given mRNA, which in turn triggers 3'-to-5' degradation by an exoribonuclease (Exo). Figure taken from Vogel & Luisi (2011).

1.4. Small RNAs in *P. aeruginosa*

sRNAs play important regulatory roles in *P. aeruginosa*. The few sRNAs functionally characterized are known to be involved in the regulation of virulence genes (RsmY/Z/W, ErsA, Sr0161 and Sr006), carbon catabolite repression (CrcZ), iron metabolism (PrrF1, PrrF2, PrrH), nitrogen assimilation (NrsZ), as well as in quorum sensing (QS) regulation (PhrS) and antibiotic resistance (ErsA and Sr0161) (Wilderman *et al*., 2004; Brencic *et al*., 2009; Sonnleitner *et al*., 2009; Oglesby-Sherrouse & Vasil 2010; Sonnleitner *et al*., 2011; Wenner *et al*., 2013). These sRNAs belong either to the group of base-pairing sRNAs or to the group of sequestering sRNAs.

PhrS, ErsA and Sr0161 are examples of *Pseudomonas* base-pairing sRNAs. Under anaerobic conditions, expression of the Anr controlled *phrS* gene leads to indirect expression of the *pqsR* gene. Hereby, PhrS stimulates translation of an upstream open reading frame to which *pqsR* is translationally coupled (Sonnleitner *et al.*, 2011). PqsR is one of the key quorum sensing regulators in *P. aeruginosa*, therefore PhrS provides a regulatory link between oxygen availability and quorum sensing. ErsA, whose transcription is σ^{22} dependent, negatively regulates *algC* translation in an Hfq-dependent manner (Ferrara *et al.*, 2015). Moreover, ErsA and Sr0161 negatively regulate *oprD* mRNA translation (Zhang *et al.,* 2017). OprD is responsible for the entry of basic amino acids, peptides and carbapenem antibiotics (Trias & Nikaido, 1990).

In *P. aeruginosa*, RsmZ/RsmY and CrcZ are members of the group of sequestering sRNAs. The RsmZ/RsmY RNAs are induced by the two-component system (TCS) GacS/GacA (Lapouge *et al*., 2008; Brencic *al.*, 2009; Goodman *et al.*, 2009). The Gac/Rsm cascade regulates the reversible switch between free-living, motile cells and sessile, non-motile cells engaged in biofilms (Chambonnier *et al.*, 2016). The regulatory RNA CrcZ is part of the CbrA/CbrB cascade and is involved in post-transcriptional regulation of carbon catabolite repression (CCR) (Sonnleitner *et al*., 2011; Sonnleitner & Bläsi, 2014). In addition to its role in CCR, CrcZ also limits biofilm formation under anaerobic conditions, and its overexpression leads to enhanced antibiotic sensitivity (*e.g.* towards fosfomycin) by cross-regulating Hfq-dependent physiological processes (Pusic *et al.*, 2016; Pusic *et al*., 2018).

1.4.1 CrcZ synthesis and stability

In *P. aeruginosa* the TCS CbrA/CbrB is required for *crcZ* transcription and contributes to maintainance of the carbon/nitrogen balance (Nishijyo *et al*., 2001; Li & Lu, 2007; Sonnleitner *et al*., 2009). The CbrB protein is a transcriptional activator for the σ^{54} RNA polymerase and belongs to the NtrC family of response regulators (Nishijyo *et al*., 2001). The genes encoding the TCS CbrA/CbrB are located upstream of the *crcZ* gene (Sonnleitner *et al*., 2009). The recognition sites for CbrB have been identified in the upstream region of the *crcZ* promoter (Abdou *et al.*, 2011). When a non-preferred substrate, *e. g.* mannitol, is used as a sole carbon source by *P. aeruginosa*, CbrA is activated by an unknown trigger, leading to

CbrA auto-phosphorylation. Upon activation, CbrA transfers the phosphoryl group to its cognate response regulator CbrB. The activated CbrB protein, assisted by the integration host factor (IHF), induces σ^{54} (RpoN)-dependent transcription of the *crcZ* gene (Sonnleitner *et al*., 2009; Abdou *et al*., 2011; Moreno *et al.*, 2012). The type of carbon source affects the activity of CbrA/CbrB, and thus the CrcZ levels, which is low in the presence of a preferred carbon source (*e.g.* succinate), is increased in the presence of an intermediate substrate (*e.g.* glucose), and is high in the presence of a non-preferred carbon source (*e.g.* mannitol) (Sonnleitner *et al*., 2009; Valentini *et al*., 2014). Although the signal activating the TCS CbrA/CbrB remains unknown, it might be related to the energy status of the cell (Valentini *et al.*, 2014).

A putative binding motif for the anaerobic master regulator Anr was identified upstream of the RpoN-dependent *crcZ* promoter, and it was shown that *crcZ* is poorly expressed in an *anr* deletion mutant (Pusic *et al*., 2016). Moreover, a RpoN binding motif was identified upstream of the *anr* coding sequence, and there is some evidence that RpoN plays a role in *anr* transcription in PAO1 during anaerobiosis. Therefore, RpoN may regulate *crcZ* expression not only directly, but also indirectly by contributing to *anr* transcription under anoxic conditions (Pusic *et al*. in preparation). Furthermore, Hfq and the catabolite repression control protein Crc also affect transcription, processing and stability of the sRNA CrcZ in *Pseudomonas putida*. By forming a complex with CrcZ, these proteins protect CrcZ from degradation by RNases (Hernández-Arranz *et al.*, 2016).

1.5 Post-transcriptional regulation of carbon catabolite repression (CCR)

Pseudomonas aeruginosa is a metabolically versatile bacterium that can utilize numerous carbon sources, which enables its survival under diverse environmental conditions. In Bacteria, the uptake and assimilation of carbon compounds is controlled by carbon catabolite repression (CCR), a regulatory mechanism that prevents the utilization of less preferred carbon sources until the preferred one is consumed (Rojo, 2010; Sonnleitner & Bläsi, 2014; Sonnleitner *et al*., 2018). Liu (1952) initially observed catabolite repression in *P. aeruginosa* through the finding that the addition of succinate or citrate blocked glucose degradation. Subsequent studies showed that in most studied *Pseudomonas spp.* the presence of organic acids (for example succinate) results in catabolite repression of degradative pathways for sugars, amino acids, and other carbon sources (Smyth & Clarke, 1975; Rojo & Dinamarca, 2004).

CCR has been most extensively studied in the model organisms *Escherichia coli* and *Bacillus subtilis* (Deutscher, 2008; Görke & Stülke, 2008; Rojo, 2010). Interestingly, the molecular mechanisms of CCR vary in these two types of bacteria. In *E. coli*, CCR is mediated by the inhibition of transcriptional activation of catabolic genes in the presence of glucose, whereas in *B. subtilis*, CCR is mediated by negative regulation through a repressor protein in the presence of glucose. In *E. coli* the major player of this regulation is the transcriptional activator CRP (cyclic AMP receptor protein), which acts in conjunction with cAMP. In

contrast, in *B. subtilis* CCR is mediated by the transcriptional repressor CcpA (catabolite control protein A). In both organisms the signal-transduction pathway regulating CCR is the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) (Görke & Stülke, 2008).

In contrast to *Enterobacteriaceae* and *Firmicutes* (Görke & Stülke, 2008), in *Pseudomonas* CCR is regulated at the post-transcriptional level and involves the RNA chaperone Hfq, the catabolite repression control protein Crc and the regulatory RNA CrcZ (Rojo, 2010; Sonnleitner & Bläsi, 2014). During growth on succinate (CCR), Hfq acts in concert with Crc as a translational repressor of catabolic genes by binding with its distal face to A-rich sequences within or adjacent to ribosome binding sites of the target mRNAs (Sonnleitner & Bläsi, 2014). Crc appears to enhance the lifetime of Hfq/Crc/RNA repressive complexes by interacting with both Hfq and RNA (Sonnleitner *et al*., 2018; Kambara *et al*., 2018; Pei *et al.,* 2019). Upon relief of CCR, *e.g.* after exhaustion of succinate and in the presence of a less preferred C-source, the levels of the Hfq-binding regulatory RNA CrcZ increase (Sonnleitner *et al*., 2009; Sonnleitner & Bläsi, 2014). CrcZ contains six A-rich sequences which can bind to the distal site of Hfq (Sonnleitner & Bläsi, 2014). By interacting with Hfq, CrcZ acts as a decoy to abrogate Hfq-mediated translational repression of catabolic genes (Figure 4) (Sonnleitner & Bläsi, 2014).

In *P. aeruginosa*, CCR not only impacts on metabolic regulation, but is also involved in complex behavior including biofilm formation, quorum sensing, virulence and antibiotic susceptibility (Yeung *et al*., 2011; Zhang *et al*., 2012).

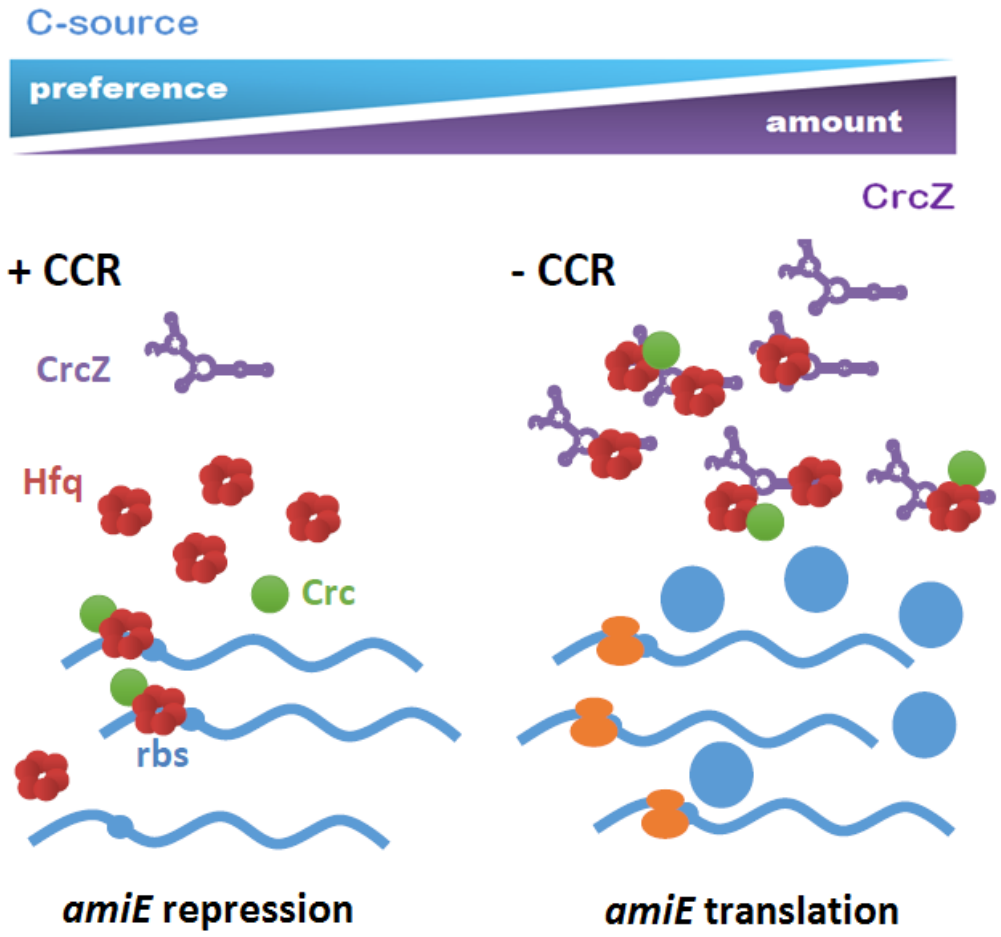

Figure 4. Simplified model for post-transcriptional regulation of CCR in *Pseudomonas aeruginosa*. During growth on a preferred carbon source, *e. g.* succinate (+CCR; left), CrcZ (in purple) is poorly transcribed and Hfq (red hexamer) in concert with Crc (in green), represses the translation of catabolic genes (*e.g. amiE*). Upon relief of CCR during growth on a non-preferred carbon source, *e. g.* amides, (-CCR; right), the level of the Hfq-binding RNA CrcZ increases, which allows translation of catabolic genes. Figure adapted from Sonnleitner & Bläsi (2014).

1.6 Metabolic regulation of biofilm formation and antibiotic susceptibility in *P. aeruginosa*

1.6.1 Hfq enhances biofilm formation of *P. aeruginosa*

Biofilm formation of *P. aeruginosa* is a highly regulated process that proceeds through several distinct stages; 1) initial attachment, 2) irreversible attachment, 3) microcolony formation, 4) macrocolony formation, and 5) dispersal. The c-di-GMP signaling pathway (Valentini & Filloux, 2016) and the Gac/Rsm network (Mikkelsen *et al.*, 2011) regulate the switch between planktonic and sessile lifestyle.

The cyclic di-GMP (c-di-GMP) second messenger is an intracellular signaling molecule that triggers the switch between planktonic and sessile lifestyles (Römling *et al.*, 2013; Jenal *et al.*, 2017; Ciofu & Tolker-Nielsen, 2019). High c-di-GMP levels correlate with biofilm

formation, whereas low levels are associated with motility in several bacteria species, *e.g. E. coli*, *P. aeruginosa*, and *Salmonella enterica* serovar Typhimurium (Simm *et al*., 2004). The levels of c-di-GMP in the cell change according to the rate of its synthesis and degradation. Cyclic di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGCs) and is degraded into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) and/or GMP by phosphodiesterases (PDEs) (Valentini & Filloux, 2016). At least five DGCs have been described to specifically control biofilm formation: WspR, SadC, RoeA, SiaD, and YfiN/TpbB (Hickman *et al*., 2005; Malone *et al*., 2010; Bernier *et al*., 2011). In contrast, the GcbA and NicD DGCs and the DipA (Pch), RbdA, and NbdA PDEs have been linked to biofilm dispersal (Ueda & Wood, 2009; Basu Roy & Sauer 2014; Petrova *et al*., 2014) (Figure 5).

Pusic *et al*. (2018) have recently provided evidence that Hfq affects the c-di-GMP levels in *P. aeruginosa*.

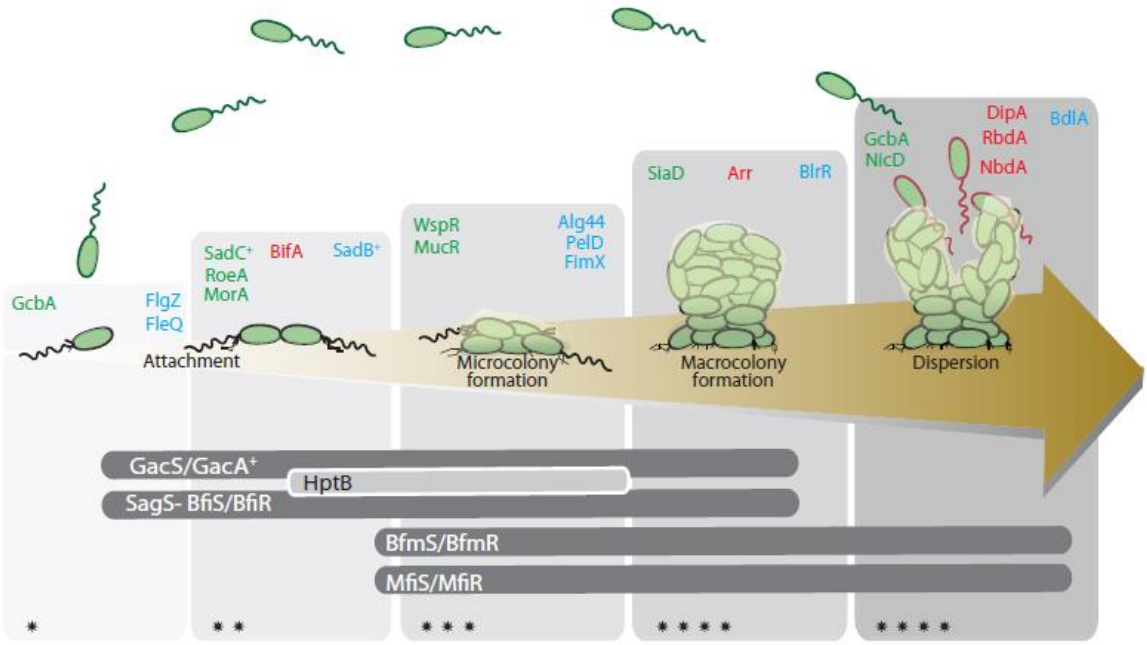

Figure 5. C-di-GMP signaling pathways in concert with two-component systems control biofilm formation in *P. aeruginosa*. Biofilm formation is a developmental process that includes attachment to and movement on the surface, formation of microcolonies, maturation, and ultimately dispersal. Planktonic, biofilm, and dispersed cells are represented by green, black, and a red outline, respectively. The upper panel illustrates DGC (green), PDE (red), and c-di-GMP receptors/effectors (blue) and the developmental stage in which they are proposed to act. The lower panel illustrates biofilm stage-specific two-component regulatory systems. The gradient of the gray panels in the background of the figure indicates increasing intracellular c-di-GMP levels (indicated with *, **, ***, and ****). Figure taken from Valentini & Filloux (2016).

The TCS GacS/GacA is known as a regulator of virulence and biofilm formation (Parkins *et al*., 2001) (Figure 6). In *P. aeruginosa*, the response regulator GacA positively controls the expression of two functionally redundant sRNAs termed RsmZ and RsmY (Lapouge *et al*., 2008; Brencic *et al.*, 2009; Goodman *et al.*, 2009). The role of these two regulatory small RNAs (sRNAs) is to titrate the translational repressor protein RsmA (Lapouge *et al*., 2008). RsmA translationally represses the genes required for chronic infections (*e.g.* genes coding for proteins involved in secondary metabolism, biofilm formation and virulence), and promotes the expression of genes needed for acute infections (*e.g.* genes coding for proteins of type III secretion and flagellar motility) (Brencic & Lory, 2009). Thus, sequestration of RsmA by RsmZ and RsmY results in de-repression of RsmA target mRNAs (Pessi *et al.*, 2001; Heeb *et al.*, 2002; Kay *et al.*, 2006; Brencic & Lory, 2009), and consequently in the switch between acute and chronic infection phenotypes. Moreover, RNase G (the *cafA* gene product) specifically cleaves RsmZ in *P. aeruginosa* biofilms**.** The expression of *cafA* is controlled by the TCS BfiS/R (Petrova & Sauer 2010) (Figure 6).

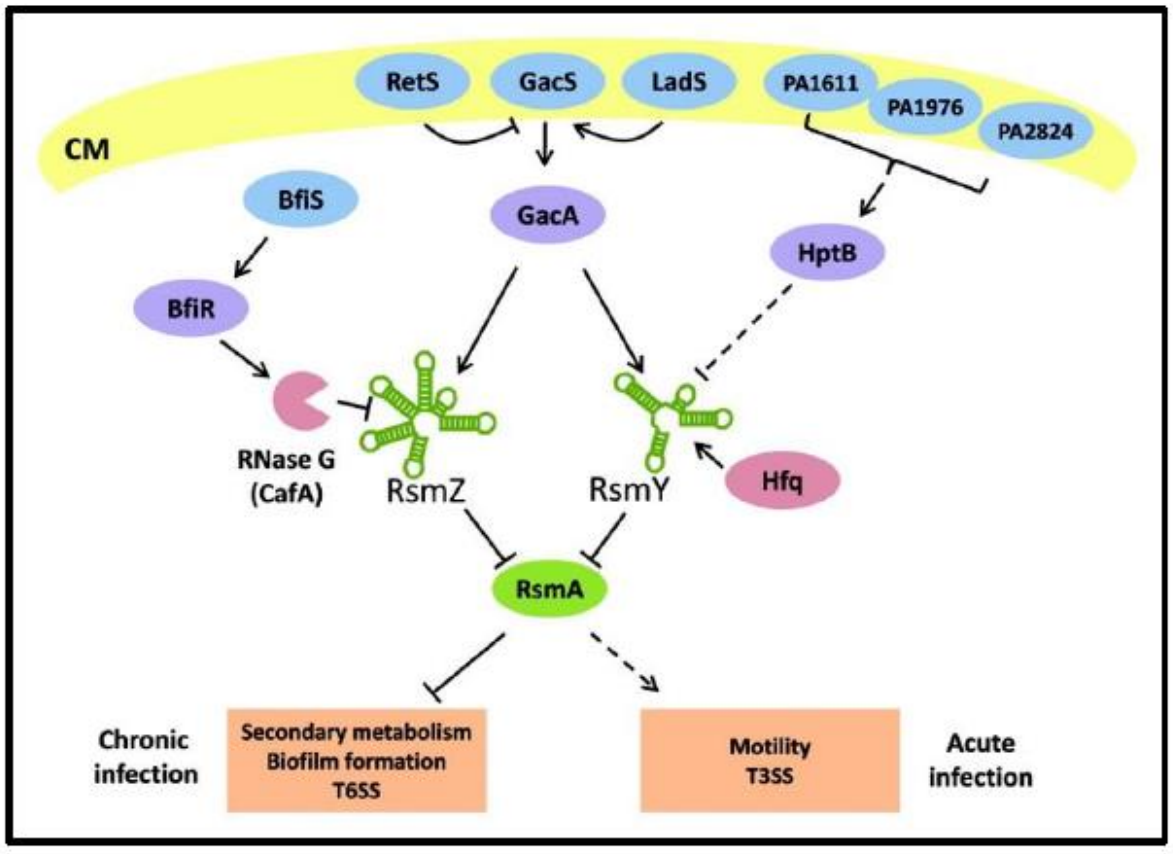

Figure 6. The Gac/Rsm pathway in *P. aeruginosa*. The TCS GacS/GacA activates transcription of the sRNAs RsmZ and RsmY. These two regulatory sRNAs titrate the translational repressor protein RsmA, which mediates the switch from an acute to a chronic infection. Hfq contributes to the regulatory cascade by stabilizing RsmY. ↓: positive control; ⊥: negative control; dashed lines: indirect control. Taken from Sonnleitner *et al.* (2012).

In *P. aeruginosa*, Hfq plays also a role in the Gac/Rsm regulatory network. Hfq was shown to bind to and stabilize the regulatory RNA RsmY, but not RsmZ (Sonnleitner *et al*., 2006). The binding can occur concurrently with RsmA and protects RsmY from RNaseE cleavage (Sorger-Domenigg *et al*., 2007) (Figure 6). As mentioned above, the Hfq is involved in both Gac/Rsm and c-di-GMP regulatory networks (Sonnleitner *et al*., 2006; Pusic *et al*., 2018). Evidence that the Gac system and c-di-GMP signaling are interlinked stems from the observation that the deletion of the RetS sensor kinase (Figure 6), which is involved in negative regulation of GacS, leads to increased c-di-GMP levels (Moscoso *et al*., 2011). Moreover, the c-di-GMP-induced T3SS/T6SS switch is dependent on the two sRNAs RsmY and RsmZ (Moscoso *et al*., 2011). In addition, Moscoso and colleagues showed that the activity of the SadC-DGC is directly responsible for the hyperbiofilm phenotype, and that elevated levels of c-di-GMP are observed in a *retS* mutant (Moscoso *et al*., 2014).

In most bacteria, Hfq positively regulates biofilm production (Martínez & Vadyvaloo, 2014). Hfq acts to increase biofilm formation in uropathogenic *E. coli, Salmonella enterica* typhimurium, *Vibrio cholerae*, *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia*, *Erwinia amylovora*, and *Vibrio alginolyticus*. In these pathogens, the respective *hfq* deletion strain not only results in reduced biofilm formation but also in pleiotropic phenotypes, including defects in quorum sensing, motility, antibiotic susceptibility, growth rate, stress tolerance, and virulence (Hammer & Bassler, 2007; Kulesus *et al*., 2008; Kint *et al*., 2010; Wu *et al*., 2010; Liu *et al*., 2011; Monteiro *et al*. 2012; Zeng *et al*., 2013). Similarly, a deletion of the *hfq* gene in PAO1 resulted in pleiotropic effects on growth and virulence (Sonnleitner *et al*., 2003). In addition, Pusic *et al*. showed that Hfq might directly or indirectly control functions implicated in anoxic biofilm formation (*e.g.* QS, glycerol metabolism and NADH/NAD+ ratios) (Pusic *et al*., 2016).

It was previously shown that a mutation of the *crc* gene in *P. aeruginosa* PA14 impairs biofilm formation and type IV pilus-mediated twitching motility (O'Toole *et al*., 2000), and that a *crc* mutant of *P. aeruginosa* PAO1 is defective in type III secretion, motility and expression of quorum sensing-regulated virulence factors (Linares *et al*., 2010). Based on a bioinformatic approach, Browne *et al*. (2010) showed that the production of some virulence traits (*e.g.* alginate, rhamnolipid) might be regulated by CCR. In addition, translation of *estA* was shown to be under CCR control (Sonnleitner & Bläsi, 2014). The *estA* gene encodes an autotransporter protein with esterase activity that is required for rhamnolipid production (Wilhelm *et al*., 1999; Wilhelm *et al*., 2007). Rhamnolipids are surface-active molecules involved in biofilm fluidity (Davey *et al*., 2003).

The deletion of the *crcZ* gene resulted in increased biofilm formation and it was suggested that CrcZ indirectly limits biofilm formation by titrating Hfq (Pusic *et al*., 2016). In anoxic biofilms, the levels of CrcZ are strongly increased compared to planktonically grown cultures. Thus, the CrcZ-mediated sequestration of Hfq increases, which leads to the cessation of further biofilm formation (Pusic *et al*., 2016).

1.6.2 Hfq increases the antibiotic susceptibility of *P. aeruginosa*

Although Hfq has been established as an important virulence factor in bacterial pathogens, there is limited knowledge on the impact of Hfq on antibiotic susceptibility (Hansen & Kaper, 2009; Yamada *et al*., 2010; Kim *et al*., 2015). Drug resistance in Bacteria is often associated with multidrug efflux pumps that decrease cellular drug accumulation (Nikaido, 1996; Zgurskaya & Nikaido, 2000). In *E. coli*, Hfq mediates drug resistance by regulating at the post-transcriptional level the production of AcrB, which is a component of the ArcAB-TolC efflux pump (Yamada *et al*., 2010). ArcAB-TolC confers resitance to quinolones *(e.g.* ciprofloxacin) and other antimicrobials (Yu *et al*., 2003; Hooper & Jacoby, 2015). The sRNA RyeB, which impacts on the expression of *tolC*, requires Hfq to mediate antibiotic resitance to quinolones (*e.g.* levofloxacin and norfloxacin) and tetracycline in *E. coli* (Kim *et al*., 2015). Moreover, the SmvA drug efflux system contributes to the Hfq-mediated drug resitance of *Salmonella* (Hayashi-Nishino *et al*., 2012). In addition, porins may also play a significant role in resistance mechanisms. In *P. aeruginosa*, the metal-specific TCS CzcRS is required for the repression of the *oprD* porin gene, which is involved in carbapenem resistance. Hfq is a key factor required for the transcriptional repression of OprD upon Zn treatment by mediating the binding of CzcR to the *oprD* promoter (Ducret *et al*., 2016). Furthermore, two *trans*-acting sRNAs (Sr0161 and ErsA) control meropenem resistance by binding to the 5′ UTR of *oprD* (Zhang *et al*., 2017). In *E. coli*, the outer membrane porin gene *ompF* is post-transcriptionally regulated by the sRNA MicF, which mediate resistance to cephalosporins (Delihas & Forst, 2001; Kim *et al*., 2015).

Recently, Pusic *et al*. (2018) showed that *P. aeruginosa hfq* deletion mutants were more susceptible to different classes of antibiotics under aerobic conditions. Deletion of *hfq* affected different mechanisms known to be involved in antibiotic susceptibility that encompass import and efflux, cell wall and LPS composition, energy metabolism as well as c-di-GMP levels. Moreover, the cross-regulation by the regulatory RNA CrcZ, which titrates Hfq, enhanced the sensitivity toward certain antibiotics such as gentamicin. Therefore, it has been proposed to (re)sensitize *P. aeruginosa* to different classes of antibiotics by controlling the synthesis of CrcZ (Pusic *et al*., 2018).

1.7 Aims of the project

Owing to Hfq sequestration, the regulatory RNA CrcZ not only enhances the suscebtibility towards antibiotics under aerobic conditions (Pusic *et al*., 2018), but also decreases biofilm formation under anoxic conditions (Pusic *et al*., 2016) in *P. aeruginosa*. The expression of *crcZ* directly correlates with the hierarchical utilization of carbon sources under aerobic (Sonnleitner *et al*. 2009; Valentini *et al*., 2014), as well as anaerobic conditions (Maximilian Bauer, unpublished data). A central goal of this study was to investigate the regulation of *crcZ* expression during anaerobiosis, and to test whether the induced synthesis of CrcZ *via* non-preferred carbon sources could be harnessed to increase antibiotic susceptibility of *P. aeruginosa* not only in planktonic cultures (Pusic *et al*., 2018), but also in anoxic biofilms.

2. Materials and methods

2.1 Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and oligonucleotides used in this study are listed in Tables 2, 3 and 4, respectively.

The cell cultures were grown overnight in Basal Salt medium (BSM) supplemented with 40 mM succinate (Sonnleitner *et al.*, 2009) (BSM-succinate) or synthetic cystic fibrosis medium (SCFM) (Palmer *et al.*, 2007) supplemented with 100 µM $FeSO_4$ (Tata *et al.*, 2016). For anaerobic nitrate respiration during anoxic growth, the BSM-succinate medium was supplemented with 100 mM KNO_3 (BSM_{anox}) and the concentration of KNO_3 of the SCFM medium was increased to 100 mM ($SCFM_{anox}$). After overnight growth the cells were diluted to an OD_{600} of 0.1 with BSM_{anox} or $SCFM_{anox}$ media, respectively, and grown anaerobically at 37°C in an anaerobic chamber fumigated with N2 (98%) and H2 (2%). To allow the growth of the glutamine auxotroph strain PAO1Δ*rpoN*, 0.2% glutamine was added to all media (Totten *et al*., 1990). The *E. coli* strain DH5α was used for the construction of plasmids. When required, the following concentrations of antibiotics were used: 100 µg/ml ampicillin and 25 µg/ml tetracycline for *E. coli*; 100 µg/ml of tetracycline for *P. aeruginosa*.

Table 2. Bacterial strains

Strains	Genotype/relevant characteristics	Sources/references
P. aeruginosa		
PAO1	Wild type	Holloway *et al*., 1979
PAO1Δ*cbrB*	PA06711	Sonnleitner *et al*., 2009
PAO1Δ*rpoN*	PAO6358	Heurlier *et al*., 2003
PAO1Δ*hfq*	in-frame deletion of *hfq*	Sonnleitner *et al*., 2017
PAO1Δ*anr*	PAO6261	Ye *et al*., 1995
E. coli		
DH5α	*recA1 endA1 hsdR17 thi-1 supE44 gyrA96 relA1 deoR*Δ(*lacZYA-argF*) *U169* (Φ80*lacZ*ΔM15)	Sambrook & Russell, 2001
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(araleu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen

Table 3. Plasmids

Plasmids	Genotype/relevant characteristics	Sources/references
pME6016	Cloning vector for transcriptional *lacZ* fusions; Tc^r	Schnider-Keel *et al*., 2000
pME6016*crcZ-lacZ*	pME6016 carrying a transcriptional *crcZ-lacZ* fusion spanning nt -162 to nt +1 relative to the *crcZ* transcriptional start site	This study
pME6016 *crcZ (TT→ AA)-lacZ*	pME6016 carrying a transcriptional *crcZ-lacZ* fusion spanning nt -162 to nt +1 relative to the *crcZ* transcriptional start site, wherein the Anr motif of the *crcZ* promoter was altered TT→ AA	This study
pUC18	Cloning vector, Amp^R	Norrander *et al*., 1983
pUC18*crcZ-lacZ*	pUC18 carrying a transcriptional *crcZ-lacZ* fusion spanning nt -162 to nt +1 relative to the *crcZ* transcriptional start site	This study

Table 4. DNA oligonucleotides used in this study

Oligo-nucleotide	Sequence (5'-3')[a]	Binding region[b]	Target gene/usage
P147 Q147	ACGT**GAATTC**CACCCTGCAACCTGTTAC ACGT**CTGCAG**CCAATACATAAGCAGATG	5308425-5308453 5308558-5308586	*crcZ*, to generate a transcriptional reporter fusion
R147 S147	GAAAACCTCAACCCAaaGATTTTACTGGGT ACCCAGTAAAATCttTGGGTTGAGGTTTTC	5308521-5308550 5308521-5308550	*crcZ*, to generate site-directed mutation within the *crcZ* promoter
K3 I26	GCTGGAGTCGTTACGTGTTG CCCCACACTACCATCGGCGATGCGTCG		*crcZ* 5S rRNA Northern-blot

a. Restriction sites are highlighted in bold.

b. Base pair annotations according to Winsor *et al.*, 2016.

2.2 Construction of plasmids

Plasmid pME6016*crcZ-lacZ* encodes a transcriptional *crcZ-lacZ* fusion. The fragment (genome coordinates: 5308435-5308586) including nucleotides -162 to +1 relative to the *crcZ* transcriptional start site was amplified by PCR using chromosomal DNA of PAO1 as a template together with the oligonucleotides P147 and Q147 (Table 3). The PCR fragment was cleaved with *Eco*RI and *Pst*I and ligated into the corresponding sites of plasmid pME6016.

Plasmid pME6016*crcZ (TT → AA)-lacZ* encodes a transcriptional *crcZ-lacZ* fusion, wherein the Anr motif of the *crcZ* promoter was altered TT→ AA. The fragment (genome coordinates: 5308435-5308586) including nucleotides -162 to +1 relative to the *crcZ* transcriptional start site was amplified by PCR using chromosomal DNA of PAO1 as a template together with the oligonucleotides P147 and Q147, and R147 and S147 (Table 3), the latter of which introduced the corresponding mutations. The PCR fragment was cleaved with *EcoR*I and *Pst*I and ligated into the corresponding sites of plasmid pME6016.

Plasmid pUC18*crcZ-lacZ* encodes a transcriptional *crcZ-lacZ* fusion. The fragment (genome coordinates: 5308435-5308586) including nucleotides -162 to +1 relative to the *crcZ* transcriptional start site was amplified by PCR using chromosomal DNA of PAO1 as a template together with the oligonucleotides P147 and Q147 (Table 3). The PCR fragment was cleaved with *EcoRI* and *PstI* and ligated into the corresponding sites of plasmid pUC18.

2.3 Northern-blot analyses

5 ml polypropylene tubes were filled with 1 ml of BSM_{anox} or $SCFM_{anox}$ medium, which was inoculated with the respective strains (OD_{600} = 0.05). The cultures were then incubated for 24 hours or 48 hours at 37°C in an anaerobic chamber. The total content of the polypropylene tube was used for RNA preparation. Total RNA was extracted using the TRIzol reagent (Ambion) according to the manufacturer's instructions. The samples were DNase I treated, followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation (Pusic *et al*., 2016). The steady state levels of CrcZ and 5S rRNA (loading control) were determined by Northern-blotting using 1 µg of total RNA. The RNA samples were denatured for 10 min at 65 °C in loading buffer containing 50% formamide, separated on a 6% polyacrylamide/ 8 M urea gel, and then transferred to a nylon membrane by electroblotting. The RNAs were cross-linked to the membrane by exposure to UV-light. The membranes were hybridized with gene-specific ^{32}P-end-labeled oligonucleotides (*crcZ*: K3; 5S rRNA: I26, Table 3). The hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

2.4 β-galactosidase assays

The β-galactosidase assays were performed after anaerobic cultivation for 24 hours in BSM_{anox} medium as described by Miller (1972), except that the cells were permeabilized with 5% toluene and the β-galactosidase activities were measured at 37°C. The β-galactosidase activity was derived from two biological replicates and are presented as mean.

2.5 Biofilm assays in polystyrene tubes

Static culture biofilm assays were performed in 5 ml round bottom polystyrene tubes (Falcon). Cells were grown under anoxic conditions in BSM_{anox} and in $SCFM_{anox}$ for 24 hours or 48 hours. The biofilms were washed with water and stained with 0.1% crystal violet

solution for 10 min. The tubes were washed and air dried. The dye bound, which is proportional to the biofilm produced, was solubilized with 96% (v/v) ethanol and the absorption was photometrically measured at 595 nm (A_{595}).

2.6 Biofilm assays in microtiter plates

To determine the effect of carbon sources on biofilm formation in $SCFM_{anox}$, the static culture biofilm assays were performed in 96-well microtiter plates. Biofilms were established with 180 µl bacterial cultures grown under anoxic conditions in $SCFM_{anox}$ for 24 hours. Then, 100 µl of $SCFM_{anox}$ and 20 µl of the carbon source (40 mM succinate, 40 mM mannitol, 40 mM lactamide or 40 mM glucose) or $SCFM_{anox}$ (mock) were added to early biofilms, which are formed after 24 hours of bacterial growth. The cultures were further grown under anoxic conditions for 24 hours. The biofilms were washed with water and stained with 0.1% (w/v) crystal violet solution for 10 min. The dye bound, which is proportional to the biofilm produced, was solubilized with 96% (v/v) ethanol and the absorption at 595 nm (A_{595}) was measured in a Synergy H1 plate reader.

2.7 Determination of the minimal biofilm-eradication concentration (MBEC) by microdilution

The static culture biofilm assays were performed in 96-well microtiter plates as described above. Briefly, biofilms were established with 180 µl bacterial cultures grown under anoxic conditions in $SCFM_{anox}$ for 24 hours. Then, 20 µl of respective carbon sources (final concentrations: 40 mM succinate, mannitol or lactamide) and 100 µl of $SCFM_{anox}$ containing serial dilutions of the antibiotic (ciprofloxacin or cefepime) were added to early biofilms. The biofilms were washed with water and stained with 0.1% (w/v) crystal violet solution followed by solubilization with 96% (v/v) ethanol. The absorption at 595 nm (A_{595}) was measured in a Synergy H1 plate reader. The MBEC correspond to the lowest concentration of antibiotic required to eradicate the biofilm (Ceri *et al*., 1999).

3. Results

3.1 RpoN and CbrA/CbrB mediated transcription of *crcZ* during anaerobiosis

Under aerobic conditions, *crcZ* transcription requires the alternative sigma factor RpoN and the response regulator CbrB, which is phosphorylated by the sensor/histidine kinase CbrA (Sonnleitner *et al.*, 2009; Abdou *et al*., 2011). A previous transcriptome study showed that the *cbrA* gene is 2.5-fold up-regulated in anoxic biofilms when compared to planktonic cultures (Tata *et al*., 2016). This finding may partly explain the observed increased expression of *crcZ* under anoxic conditions (Pusic *et al*. 2016). To test whether CbrB is required for *crcZ* transcription in anoxic biofilms, the CrcZ steady-state levels were determined in strains PAO1 and PAO1Δ*cbrB* grown under anaerobic conditions for 24 hours (B24 cultures) or 48 hours (B48 cultures) in $BSM_{anox,}$ as well as $SCFM_{anox}$ medium, the latter of which approximates to the conditions in the CF lung. When compared to the corresponding wild-type strain, the CrcZ steady-state levels were diminished in the *cbrB* deletion mutant at both time points and in both media (Figure 7A and B).

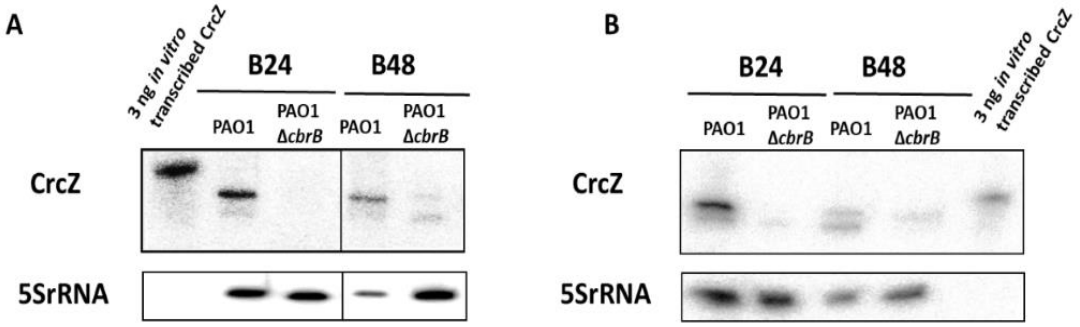

Figure 7. CbrB is required for *crcZ* transcription in anoxic biofilms. The PAO1 and PAO1Δ*cbrB* strains were grown under anoxic conditions for 24 hours (B24) and 48 hours (B48) in **(A)** BSM_{anox} medium and **(B)** $SCFM_{anox}$ medium. Total RNA was isolated, and 1 µg of total RNA was used for the Northern-blot analyses. 5S rRNA served as a loading control. *In vitro* transcribed CrcZ RNA was loaded for size comparison.

When compared with the wild-type strain a *P. aeruginosa crcZ* deletion mutant has been shown to display an increased ability to form anoxic biofilms (Pusic *et al*., 2016). Therefore, it was next examined whether the same phenotype is observed in a *cbrB* deletion mutant due to diminished levels of CrcZ. Anoxic biofilm formation was assessed by a static crystal violet assay in B24 and B48 cultures of the strains PAO1 and PAO1Δ*cbrB*, respectively, in both, BSM_{anox} medium and in $SCFM_{anox}$ medium (Figure 8A and B). Consistent with the notion, anoxic biofilm formation was considerably increased in the PAO1Δ*cbrB* strain, when compared with PAO1 in both B24 and B48 cultures grown in $SCFM_{anox}$ medium (Figure 8B) as well as in B48 cultures grown in BSM_{anox} medium (Figure 8A). However, biofilm formation was increased in the PAO1 strain (Figure 8A) in B24 cultures grown in BSM_{anox}.

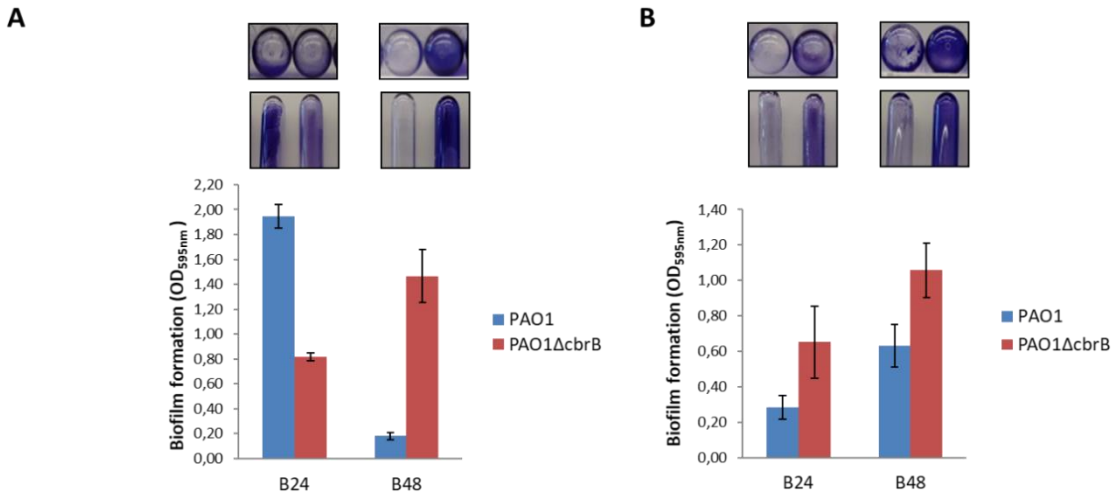

Figure 8. Biofilm formation is increased in PAO1Δ*cbrB* compared to PAO1. The PAO1 and PAO1Δ*cbrB* strains were grown under anoxic conditions in **(A)** BSM_{anox} medium and **(B) in** $SCFM_{anox}$ medium for 24 hours (B24) and 48 hours (B48). Top, biofilm formation was assessed by a crystal violet assay. Bottom, graphical representation of the results obtained with cultures of PAO1 (blue bars) and PAO1Δ*cbrB* (red bars).

3.2 Anr contributes to RpoN/CbrA/CbrB-mediated transcription of *crcZ* during anaerobiosis

Under anoxic conditions, the CrcZ steady-state levels are ~50 fold increased when compared to aerobic conditions (Pusic *et al*., 2016). A putative binding site for the anaerobic regulator Anr was identified upstream of the RpoN-dependent *crcZ* promoter (Figure 10A, in violet). Moreover, *crcZ* was poorly expressed in the absence of Anr during anaerobiosis (Pusic *et al*., 2016). To further assess how the expression of *crcZ* is regulated during anaerobiosis, we next determined β-galactosidase activities obtained with the transcriptional $crcZ_{162}$-*lacZ* reporter gene encoded by plasmids pME6016P*crcZ-lacZ* and pME6016P$crcZ_{(TT\rightarrow AA)}$-*lacZ* in strains PAO1, PAO1Δ*cbrB*, PAO1Δ*rpoN*, PAO1Δ*hfq* and PAO1Δ*anr* grown in BSM_{anox} for 24 hours (Figure 9). As expected, *crcZ-lacZ* expression was abolished in strains PAO1Δ*cbrB* and PAO1Δ*rpoN* (Figure 10B), demonstrating that CbrA/CbrB and RpoN act as the main regulators of *crcZ* expression during anaerobiosis. In contrast to PAO1, c*rcZ* transcription was also strongly diminished in the PAO1Δ*hfq* strain (Figure 10B). This result is in line with previous data indicating that Hfq impacts on *crcZ* synthesis (Hernández-Arranz *et al.*, 2016). Moreover, the c*rcZ* promoter activity was reduced in the absence of Anr, indicating that Anr contributes to *crcZ* expression during anaerobiosis (Figure 10B). For verification, a part of the putative Anr motif located upstream of the RpoN-dependent *crcZ* promoter was altered (TT→AA) in plasmid pME6016 P*crcZ-lacZ* (Figure 9A). However, the base changes resulted in reduced activity of the *crcZ* promoter in both strains, PAO1 and PAO1Δ*anr*, respectively (Figure 10B). This could indicate that the substitution TT→AA in the putative Anr motif might interfere with binding of integration host factor (IHF), the recognition motif of which overlaps with the putative Anr binding site (Figure 10A).

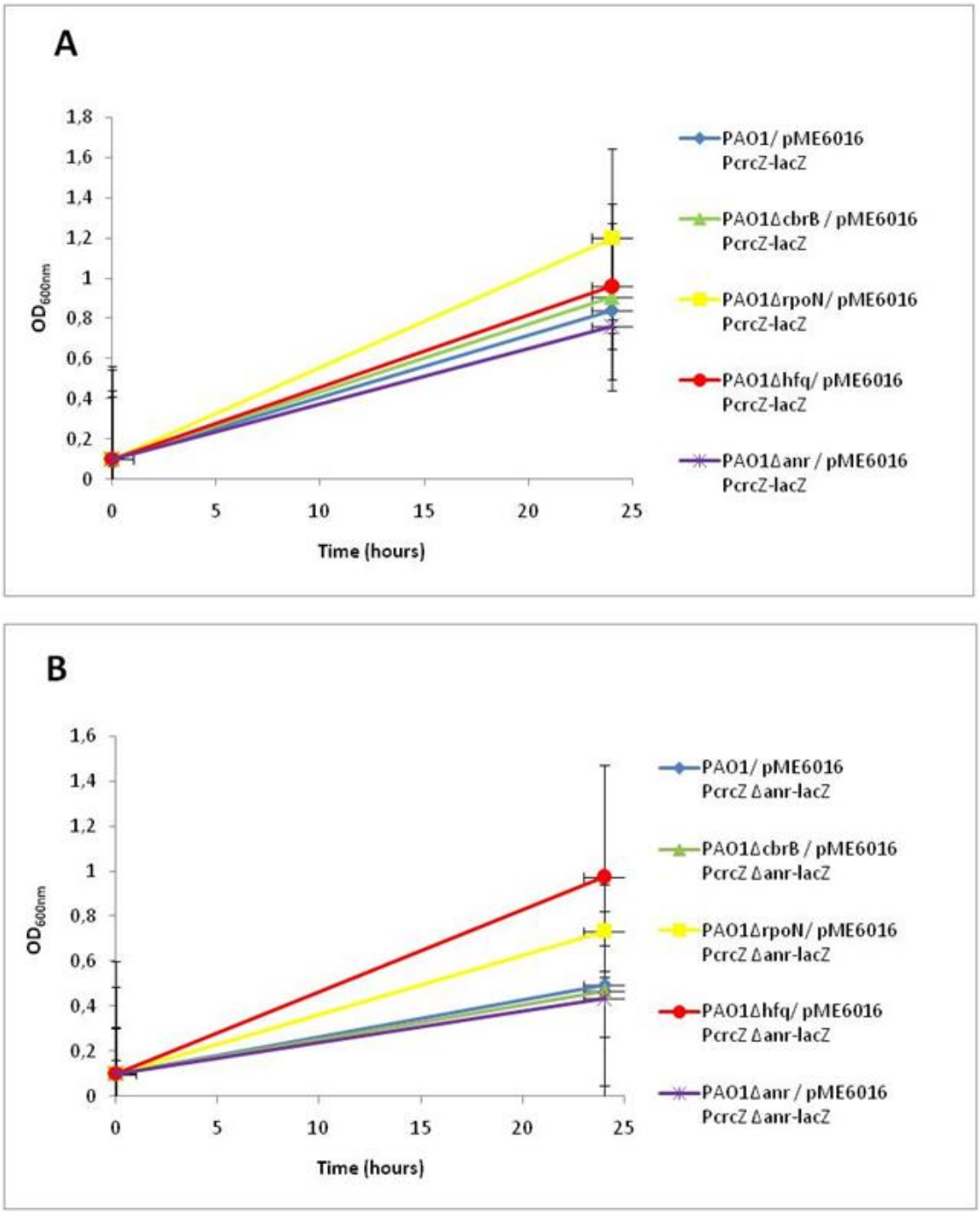

Figure 9. Growth of strains PAO1, PAO1*ΔcbrB,* PAO1*ΔrpoN,* PAO1*Δhfq and* PAO1*Δanr* for 24 hours in BSM_{anox} medium harboring plasmid pME6016P*crcZ-lacZ* (A) or plasmid pME6016P$crcZ_{(TT\rightarrow AA)}$-*lacZ* (B).

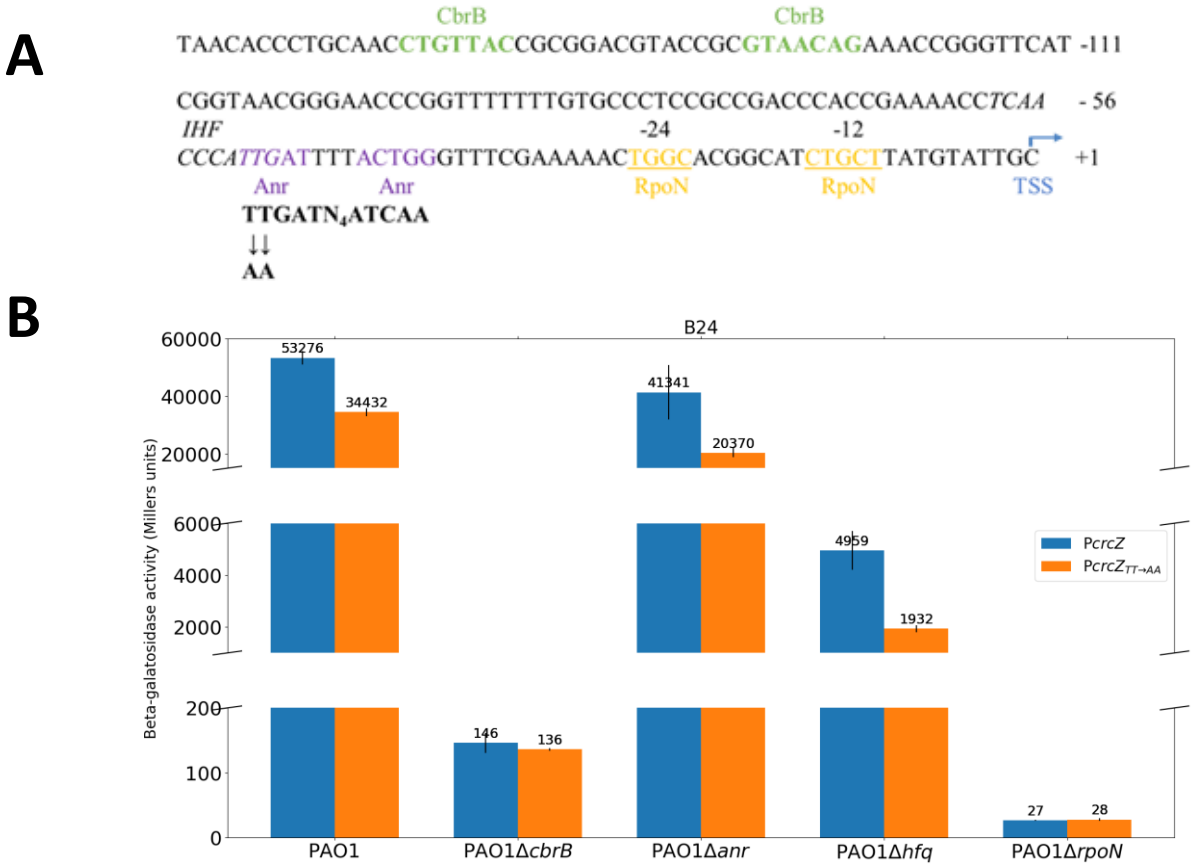

Figure 10. Anr contributes to the RpoN and CbrA/CbrB mediated expression of *crcZ* during anaerobiosis. (A) Promoter region of *crcZ*. The transcriptional start site (TSS, +1) of *crcZ* is marked with a blue arrow. The RpoN promoter motif is underlined in ochre-yellow. The putative IHF binding site is shown in italics. The CbrB binding site is denoted in green. A putative recognition motif for Anr is indicated in violet with the corresponding consensus motif shown below. The substituted bases are highlighted by black arrows. **(B)** The cultures were grown anaerobically in BSM_{anox} medium for 24 hours. The bars represent the β-galactosidase values obtained with the transcriptional *crcZ-lacZ* reporter gene encoded by plasmid pME6016P*crcZ-lacZ* in strains PAO1 (pME6016P*crcZ-lacZ*) (bar 1), PAO1 (pME6016P*crcZ* $_{(TT\rightarrow AA)}$-*lacZ*) (bar 2), PAO1Δ*cbrB* (pME6016P*crcZ-lacZ*) (bar 3), PAO1Δ*cbrB* (pME6016P*crcZ* $_{(TT\rightarrow AA)}$-*lacZ*) (bar 4), PAO1Δ*anr* (pME6016P*crcZ-lacZ*) (bar 5), PAO1Δ*anr* (pME6016P*crcZ* $_{(TT\rightarrow AA)}$-*lacZ*) (bar 6), PAO1Δ*hfq* (pME6016P*crcZ-lacZ*) (bar 7), PAO1Δ*hfq* (pME6016P*crcZ* $_{(TT\rightarrow AA)}$-*lacZ*) (bar 8), PAO1Δ*rpoN* (pME6016P*crcZ-lacZ*) (bar 9) and PAO1Δ*rpoN* (pME6016P*crcZ* $_{(TT\rightarrow AA)}$-*lacZ*) (bar 10). Error bars, mean ± s.d. of three independent experiments.

3.3 Harnessing metabolism to decrease anoxic biofilm formation and to increase antibiotic susceptibility

By titrating Hfq, CrcZ was shown to negatively impact on anoxic biofilm formation (Pusic *et al*., 2016). On the other hand, over-expression of *crcZ* enhanced the sensitivity towards antibiotics under aerobic conditions (Pusic *et al*., 2018). Since the CrcZ levels correlate with carbon source utilization under aerobic as well as anaerobic conditions (Sonnleitner *et al*. 2009; Valentini *et al*., 2014), it was next tested whether we could harness metabolism to decrease anoxic biofilms and to increase their susceptibility towards different antibiotics.

3.3.1 The addition of "poor carbon sources" during growth in SCFM does not affect the formation of anoxic biofilms in SCFM medium

In previous work from our lab, the ability of the PAO1 strain to form 96 hours-old anoxic biofilms (B-96) was assessed in BSM_{anox} medium supplemented with 40 mM of different carbon sources including histidine, lactamide, pyruvate, arginine, mannitol, lactate, glutamate, citrate, glucose and succinate (P. Pusic, unpublished data). Different carbon sources seemed to affect anoxic biofilm formation of B-96 cultures in BSM_{anox} medium as assessed by the crystal violet assay. For instance, mannitol and lactamide (non-preferred carbon sources) decreased biofilm formation while succinate (a preferred carbon source) increased it, which is in accordance with the hierarchical assimilation of the carbon sources and its impact on *crcZ* expression under aerobic conditions (Valentini *et al*., 2014).

Therefore, it was next tested whether the same outcome is obtained with cells grown in $SCFM_{anox}$ medium, which approximates to the conditions in the CF lung. The PAO1 strain was grown under anoxic conditions in $SCFM_{anox}$ for 24 hours (B24 cultures). At this stage of anoxic biofilm formation, succinate (40 mM final concentration; preferred carbon source), glucose (less preferred carbon source), mannitol and lactamide (non-preferred carbon sources), respectively, were added to a final concentration of 40 mM. Then, the cultures were grown for additional 24 hours under anoxic conditions. Anoxic biofilm formation was assessed by a static crystal violet assay. As shown in Figure 11, the addition of succinate or of non-preffered carbon sources (glucose, mannitol or lactamide) to SCFM did not affect the formation of anoxic biofilms. However, the addition of glucose caused a slight increase in the growth of the cells (Figure 12).

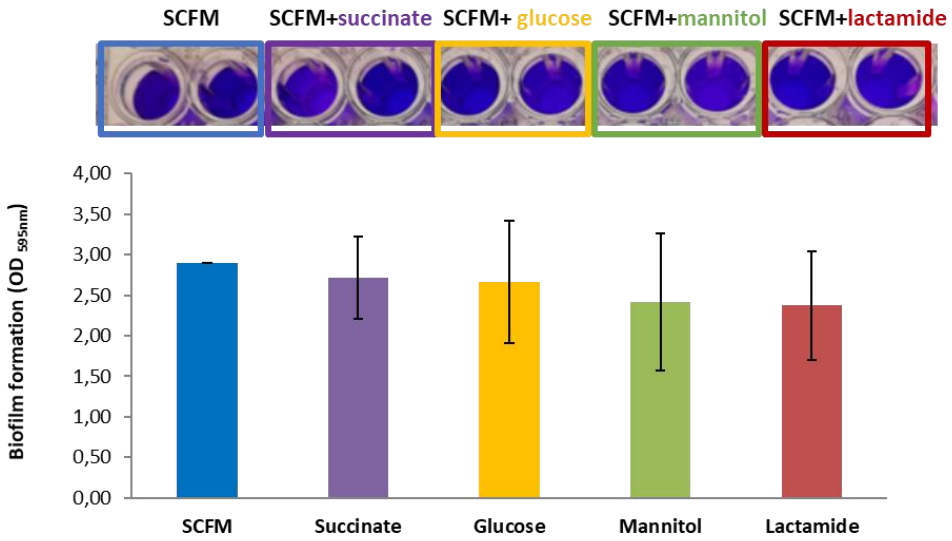

Figure 11. The addition of different carbon sources to SCFM medium does not affect anoxic biofilm formation. Top, biofilm formation was assessed by a crystal violet assay. Bottom, graphical representation of the results obtained with B48 cultures of PAO1 strain grown initially for 24 hours in $SCFM_{anox}$ medium. Then, succinate (violet bar), glucose (ochre-yellow bar), mannitol (green bar) or lactamide (red bar) were added to a final concentration of 40 mM. Mock control (blue bar), $SCFM_{anox}$ was added. Cultures were grown anoxically for additional 24 hours and biofilm formation was assessed. Error bars, mean ± s.d. from 4 biological replicates and 2 technical replicates.

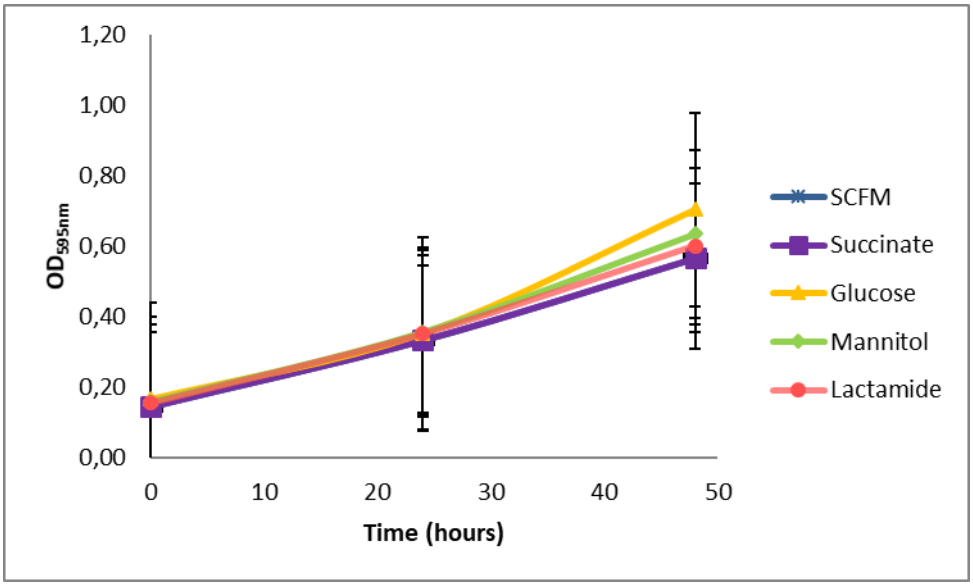

Figure 12. Impact of different carbon sources on anaerobic growth in $SCFM_{anox}$ medium. Growth curves of PAO1 grown initially for 24 hours in $SCFM_{anox}$ medium. Then, succinate (violet), glucose (ochre-yellow), mannitol (green) or lactamide (red) was added to a final concentration of 40 mM. Mock control (blue), $SCFM_{anox}$ was added. Cultures were grown anoxically for additional 24 hours. Error bars, mean ± s.d. from 4 biological replicates and 2 technical replicates.

3.3.2 Addition of non-preferred carbon sources during growth in SCFM increases the efficacy of ciprofloxacin and cefepime against anoxic biofilms

Next, it was tested whether the susceptibility of anoxic biofilms towards ciprofloxacin (CIP) and cefepime (CEF) is altered in the presence of succinate, mannitol or lactamide (Figure 13 and Figure 14). The PAO1 strain was grown under anoxic conditions in $SCFM_{anox}$ medium for 24h (B24 cultures). Then, succinate, mannitol or lactamide were added to a final concentration of 40 mM in combination with increasing concentrations of the respective antibiotic. The cultures were further grown for 24 hours under anoxic conditions (B48 cultures). Anoxic biofilm formation of B48 cultures was assessed by a static crystal violet assay (Figure 13 and Figure 14). In planktonic cultures, the minimal inhibitory concentration (MIC) is a parameter used to determine antibiotic activity. The MIC corresponds to the lowest concentration of an antibiotic that inhibits visible growth of a planktonic culture after overnight incubation. At variance, the minimal biofilm inhibitory concentration (MBIC) and the minimal biofilm-eradication concentration (MBEC) are parameters used to quantify the antimicrobial activity towards bacterial biofilms. The MBIC is defined as the lowest concentration of a drug that resulted in an $OD_{650\ nm}$ difference of ≤ 10% of the mean of two positive control well readings (Moskowitz *et al*., 2004). The MBEC is defined as the lowest concentration of antibiotic required to eradicate the biofilm (Ceri *et al*., 1999) or, in other words, the lowest concentration of an antibiotic that prevents visible growth in the recovery medium used to collect biofilm cells. To assess the activity of the tested antibiotics in combination with different carbon sources on anoxic biofilms, the MBECs were determined as described in Material and Methods. In $SCFM_{anox}$ medium without addition of any C-source, the MBEC for CIP was 4 µg/ml (Figure 13), whereas the MBEC for CEF could not be determined (Figure 14). The exposure to sub-inhibitory concentrations (between 0.5 and 2 µg/ml) of CIP induced biofilm formation (Figure 13) as previously reported (Morero *et al.*, 2011; Ahmed *et al.*, 2018). When CIP was added in concentrations between 0.5 and 2 µg/ml in combination with the non-preferred carbon source lactamide, biofilm formation decreased. In comparison, biofilm formation was much stronger in the presence of CIP when succinate was added (Figure 13). Similarly, the addition of CEF at concentrations between 128 and 512 µg/ml combined with lactamide decreased biofilm formation when compared with the considerable increase that occurred when succinate was added (Figure 14). Nevertheless, biofilm formation declined at low concentrations of CEF (32 to 64 µg/ml). This contradictory finding could be explained by an insufficient staining when high-biomass biofilms are formed (Müsken *et al*., 2010). It is noteworthy that the combination of CIP or CEF with mannitol seems not to affect biofilm formation when compared to the antibiotic alone (Figure 13 and 14). Taken together these results showed that the efficiency of both CIP and CEF on biofilm formation increased when they were added in combination with the non-preferred carbon source lactamide.

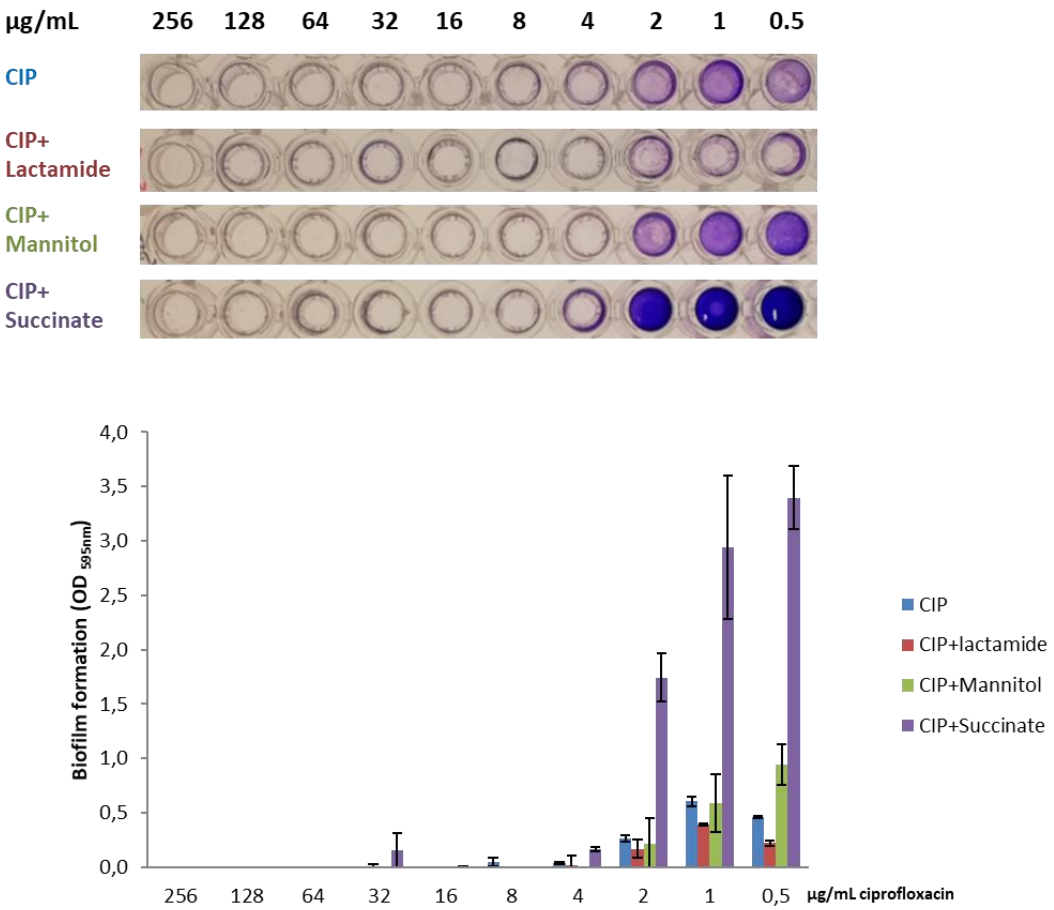

Figure 13. Carbon source dependent susceptibility of anoxic biofilms to ciprofloxacin. Top, biofilm formation was assessed by a crystal violet assay. Bottom, graphical representation of the results obtained with B48 cultures of PAO1 strain grown initially for 24 hours in $SCFM_{anox}$. Then, succinate (violet bar), mannitol (green bar) and lactamide (red bar), respectively, were added to a final concentration of 40 mM in combination with increasing concentrations of ciprofloxacin (CIP). Blue bar, $SCFM_{anox}$was added. The cultures were then grown anoxically for 24 hours and biofilm formation was assessed. Error bars, mean ± s.d. from two independent experiments.

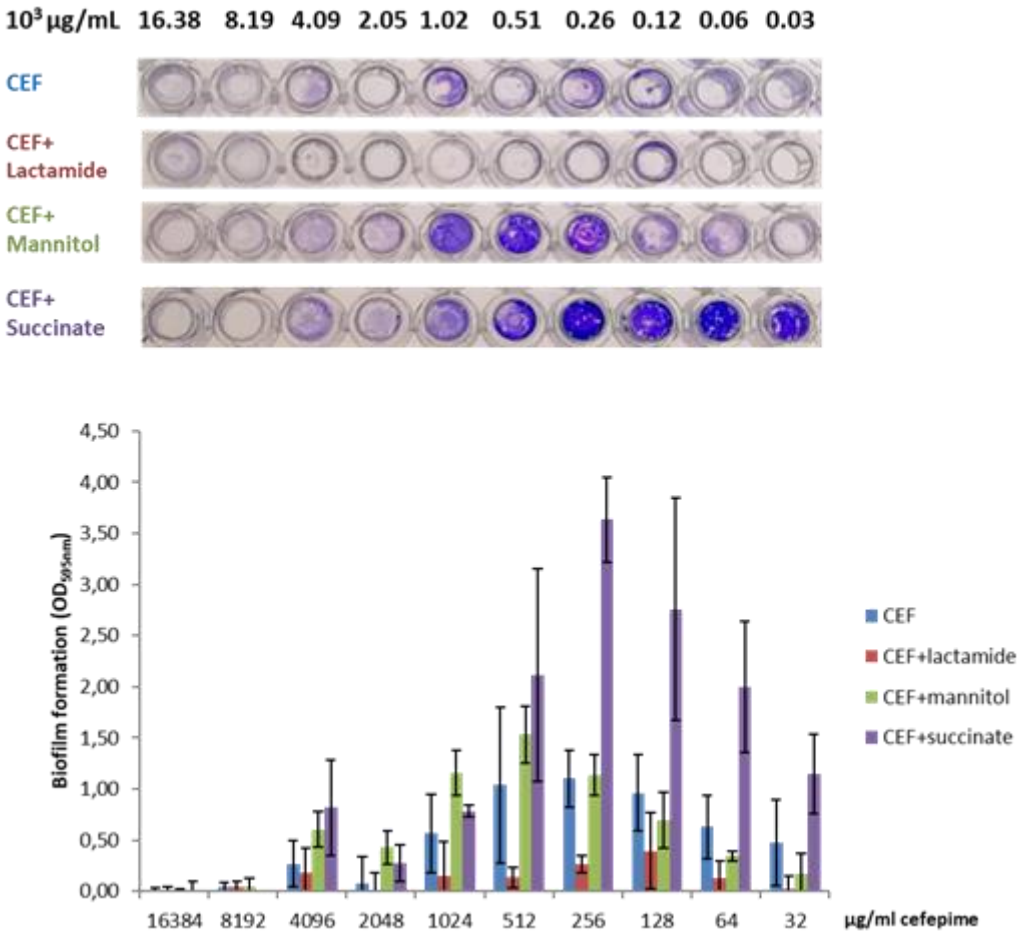

Figure 14. Carbon source dependent susceptibility of anoxic biofilms to cefepime. Top, biofilm formation was assessed by a crystal violet assay. Bottom, graphical representation of the results obtained with B48 cultures of PAO1 strain grown initially for 24 hours in $SCFM_{anox}$ medium. Then, succinate (violet bar), mannitol (green bar) and lactamide (red bar), respectively, were added to a final concentration of 40 mM in combination with increasing concentrations of cefepime (CEF). Blue bar, $SCFM_{anox}$was added. The cultures were then grown anoxically for 24 hours and biofilm formation was assessed. Error bars, mean ± s.d. from three independent experiments.

4. Discussion

4.1 Regulation of *crcZ* expression during anaerobiosis

In *P. aeruginosa* the *cbrA*/*cbrB* genes are situated upstream of the *crcZ* gene (Sonnleitner *et al*., 2009), and the binding sites for the CbrB response regulator have been mapped in the upstream region of the *crcZ* promoter (Abdou *et al.*, 2011). It has been shown that CbrA/CbrB and the alternative sigma factor RpoN are required for *crcZ* transcription under aerobic conditions (Sonnleitner *et al*., 2009; Abdou *et al*., 2011). However, whether this is also the case during anaerobiosis has so far not been studied. Previous studies have shown that the *cbrA* transcript is 2.5-fold more abundant in anoxic biofilms than in planktonic cultures (Tata *et al*., 2016), and the levels of CrcZ were found to be ~ 50-fold elevated during anaerobiosis when compared with aerobic conditions (Pusic *et al*., 2016). On the other hand, the absence of CrcZ in *P. aeruginosa* correlated with an increased ability to form anoxic biofilms (Pusic *et al*., 2016). I therefore first asked whether the CrcZ steady-state levels are affected by the presence/absence of CbrB during anaerobiosis, and whether this might impact on anoxic biofilm formation. The results showed that in BSM and SCFM media, the CrcZ steady-state levels were strongly decreased in the *cbrB* deletion mutant under anaerobic conditions when compared to the wild-type strain (Figure 7), The observed decreased steady-state levels of CrcZ in *cbrB* mutants were further supported by the decreased β-galactosidase activities obtained with the transcriptional P*crcZ-lacZ* reporter gene in the absence of CbrB (Figure 10B). In summary, these studies revealed that the TCS CbrA/CbrB is also required for anoxic *crcZ* synthesis.

As anticipated from the diminished levels of CrcZ, the *cbrB* deletion mutant formed also more anoxic biofilms (Figure 8), except for the B24 cultures grown in BSM_{anox} wherein the *cbrB* deletion mutant produced less anoxic biofilms than the PAO1 strain (Figure 8A). Crc, which is part of a signal transduction pathway that can sense and respond to nutritional cues (O'Toole *et al*., 2000), is involved in the transition from planktonic to biofilm growth by playing a role in the control of genes required for extracellular appendages (*e.g.* flagella and type IV pili) that mediate flagellar and twitching motilities in order to initiate surface colonization (O'Toole *et al*., 2000; Vallet *et al*., 2004). It seems interesting to test whether these genes are stronger expressed during early anoxic biofilm formation in BSM_{anox} when compared to $SCFM_{anox}$. This approach might reveal why the wild-type strain produced more anoxic biofilms in B24 cultures grown in BSM_{anox} when compared with the *cbrB* deletion mutant.

In addition, various studies have shown the role of other factors in the regulation of *crcZ* expression. A putative motif for the anaerobic global regulator Anr was identified upstream of the RpoN-dependent *crcZ* promoter, overlapping with the integration host factor (IHF) recognition motif (Abdou *et al.*, 2011; Figure 10A). Moreover, a putative RpoN binding motif was identified upstream of the *anr* coding sequence, which may suggest that RpoN is as well

a crucial player in *anr* transcription in PAO1 during anaerobiosis. This notion is supported by a recent finding that RpoN inhibition negatively impacts the expression of *anr* and Anr-dependent genes (Lloyd *et al.*, 2017). Consequently, RpoN may regulate *crcZ* expression not only directly, but also indirectly by contributing to *anr* transcription under anoxic conditions (Pusic *et al*., in preparation).

To assess the role of RpoN and Anr on *crcZ* expression during anaerobiosis, the β-galactosidase activities conferred by the transcriptional *crcZ*-lacZ reporter gene were assessed in PAO1 and in the deletion-mutants PAO1Δ*rpoN* and PAO1Δ*anr*. The β-galactosidase activity was drastically reduced in strain PAO1Δ*rpoN* (Figure 10B), showing that the alternative sigma factor RpoN plays also a crucial role in the regulation of *crcZ* expression under anoxic conditions.

In the absence of Anr, *crcZ* was poorly expressed during anaerobiosis (Pusic *et al*., 2016). However, the *crcZ* promoter activity was not drastically reduced in strain PAO1Δ*anr* (Figure 10B). In contrast, the alteration of the putative Anr motif (TT→AA; Figure 10A) led to the reduction of the *crcZ* promoter activity in strain PAO1 (pME6016P*crcZ*$_{(TT\rightarrow AA)}$-*lacZ*), as well as in strain PAO1Δ*anr* (pME6016P*crcZ* $_{(TT\rightarrow AA)}$-*lacZ*; Figure 10B). This result might be explained by the fact that the substitution TT→AA in the putative Anr motif can interfere with binding of the integration host factor (IHF), the recognition motif of which overlaps with the putative Anr binding site (Figure 10A). Similar results were obtained in *P. putida*, not only for the Δ*cbrB* mutant but also in the *ihf* (=IHF3) background (Barroso *et al.*, 2018). CbrB and integration host factor (IHF) appear therefore to be required for *crcZ* expression in both species, *P. aeruginosa* and *P. putida*. As previously described, IHF contributes to the correct assembly of transcription initiation complexes at a number of σ^N-dependent promoters by inducing a curvature in the DNA, and assists CbrB-mediated transcription activation of *crcZ* and *crcY* (Garcia-Maurino *et al*., 2013). To further verify whether Anr is really involved in *crcZ* transcription under anaerobic conditions, another mutation in the putative Anr binding site that is not overlapping with the recognition motif of the IHF could be introduced.

To gain more information on factors that control *crcZ* expression during anaerobiosis, the β-galactosidase activity conferred by the transcriptional *crcZ*-lacZ reporter gene was also determined in the deletion mutant PAO1Δ*hfq*. Our study showed that the β-galactosidase activity of the wild type strain (PAO1) was drastically reduced in this mutant (Figure 10B). This finding provided evidence that Hfq, which is mediating CrcZ synthesis and stability (Hernández-Arranz *et al*., 2016; Sonnleitner *et al*., 2018) under aerobic conditions, is also affecting the CrcZ levels during anaerobiosis.

4.2 Harnessing metabolism to increase antibiotic susceptibility and to decrease anoxic biofilm formation

During CCR, Hfq and Crc translationally repress several catabolic genes (Sonnleitner & Bläsi, 2014; Sonnleitner *et al*., 2018). Upon relief of CCR, the regulatory RNA CrcZ titrates Hfq. It was previously shown that less preferred carbon sources induce the transcription of *crcZ* during aerobic conditions (Zhang & Rainey, 2008; Sonnleitner *et al.*, 2009) as well as under anoxic conditions (Maximilian Bauer, unpublished data). The cross-regulation by the regulatory RNA CrcZ, which sequester Hfq, enhances the sensitivity toward antibiotics (*e.g.* gentamicin and cefepime) under aerobic conditions (Pusic *et al*., 2018). Therefore, another aim was to test whether the induced synthesis of *crcZ via* non-preferred carbon sources could be harnessed to increase antibiotic susceptibility of *P. aeruginosa* not only in planktonic cultures (Pusic *et al*., 2018), but also in anoxic biofilms.

It has been shown that C-sources can affect antibiotic resistance (Barraud *et al*., 2013). Previous studies reported that stimulating metabolic activity of *E. coli* persister cells by the addition of carbon sources such as mannitol, glucose, fructose and pyruvate could restore their susceptibility to antibiotics by generating a proton-motive force (PMF) (Allison *et al*., 2011). Barraud *et al*. (2013) demonstrated that mannitol prevented the formation of persister cells and enhanced the sensitivity of *P. aeruginosa* biofilms to tobramycin. As these effects were abolished by a PMF inhibitor, and as another metabolic substrate, glucose, had similar impacts, it is likely that the primary effect is metabolic (Barraud *et al*. 2013). Meylan *et al*. (2017) provided evidence that carbon sources affect antibiotic susceptibility *via* tricarboxylic acid cycle (TCA) control. Fumarate activates cellular respiration and generates a PMF by stimulating the TCA cycle, thus potentiating tobramycin susceptibility. In contrast, glyoxylate promoted tolerance by repressing the TCA cycle. The PMF plays an important role in the function of drug efflux pumps (Du *et al*., 2018). It has been shown that a *P. aeruginosa hfq* deletion mutant displayed a reduced PMF that might impede antibiotic efflux, thus leading to increased susceptibility to certain antibiotics, such as β-lactams, fluoroquinolones, tetracycline and aminoglycosides that are exported via efflux pumps (*Pusic et al*., 2018). Besides affecting energy metabolism, Hfq also influences different mechanisms known to be involved in antibiotic susceptibility including import and efflux, cell wall and LPS composition as well as c-di-GMP levels (Pusic *et al*. 2018). Here, we found that adding non-preferred carbon sources (lactamide) during growth in SCFM increased the efficacy of ciprofloxacin and cefepime against anoxic biofilms (Figures 13 and 14). A recent study showed that the addition of OAA to a complex medium increased CrcZ levels and enhanced the susceptibility towards gentamicin and cefepime under aerobic conditions (Pusic *et al*., 2018). Thus, our results corroborate the hypothesis that the induced synthesis of CrcZ by non-preferred carbon-sources leads to sequestration of Hfq, which consequently enhances the sensitivity towards antibiotics in anoxic biofilms.

We determined the minimal biofilm eradication concentration (MBEC) for CIP and for CEF in $SCFM_{anox}$ medium without addition of any C-source. The MBEC for CIP was 4 µg/ml (Figure 13), whereas the MBEC for CEF could not be determined (Figure 14). This is in accordance with previously published data which have shown that biofilms of various clinical strains grown under aerobic conditions were responsive to ciprofloxacin, but not to meropenem and ceftazidime, which belong to carbapenems and to the third class of cephalosporins, respectively (Müsken *et al*., 2010). This can be explained by the over-production of beta-lactamases in biofilms. For example, ceftazidime is hydrolyzed by the AmpC beta-lactamase, the levels of which are increased in biofilms (Hengzhuang *et al*., 2013). Therefore, beta-lactams have to be used in high dosages (Bowler *et al*., 2012) or in combination with beta-lactamase inhibitors (such as ceftolozane/tazobactam or ceftazidime/avibactam) (Torrens *et al*., 2016) to overcome the degradative capacity of the enzymes (Ciofu & Tolker-Nielson, 2019). Although a high dosage of CEF (16384 µg/ml) was used, it was not possible to accurately determine the MBEC for CEF (Figure 14).

The formation of static biofilms increases when *P. aeruginosa* cells are incubated in the presence of sub-inhibitory concentrations of ciprofloxacin, tobramycin, or tetracycline, while no such inhibitory effect is detected with other antibiotics, such as carbenicillin, chloramphenicol, or polymyxin (Hoffman *et al*., 2005; Linares *et al*., 2006; Morita *et al*., 2014). Similarly, we found that treatment with sub-inhibitory concentrations of ciprofloxacin and cefepime increased biofilm formation (Figures 13 and 14). The underlying mechanisms could be increased eDNA release, induction of phage elements or different regulatory responses (Ranieri *et al*., 2018). At sub-inhibitory concentrations of antibiotics, the most susceptible members of a bacterial population die, leading to the release of eDNA, which results in an increased biofilm formation by the remaining cells (Ranieri *et al*., 2018). The release of eDNA can also result from the cell lysis caused by the induction of prophages (Ranieri *et al*., 2018). Furthermore, antibiotics can act, at sub-inhibitory concentrations, as signaling molecules either directly or indirectly through the induction of cell stress responses or by increase in production of secondary messengers such as cyclic-di-GMP or ppGpp that in turn modulate gene expression (Ranieri *et al*., 2018). For instance, sub-inhibitory concentrations of aminoglycosides, especially, tobramycin, induce the expression of the gene *arr*, encoding the aminoglycoside response regulator. Tobramycin, either directly or indirectly, enhances the phosphodiesterase activity of the Arr cytoplasmic EAL domain, leading to c-di-GMP inactivation and augmented biofilm formation (Hoffman *et al*., 2005). Ciprofloxacin can induce cell lysis and release of eDNA *via* the SOS response regulator RecA (Turnbull *et al*., 2016). Furthermore, in *P. aeruginosa* biofilms, the exposure to sub-inhibitory concentrations of imipenem has been shown to strongly induce the expression of *ampC*, leading to antibiotic degradation, as well as to the expression of genes (*algD* to *algA*) encoding fuctions for alginate biosynthesis, resulting in thicker and more robust biofilms (Bagge *et al*., 2004). This increased thickness and biomass may increase the

potential of β-lactamase accumulation in the biofilm, thereby reducing the effect of β-lactam treatment on *P. aeruginosa* cells growing in biofilms (Bagge *et al*., 2004).

In this study, I asked wether an increased synthesis of CrcZ would decrease anoxic biofilm formation in the presence of sub-inhibitory concentrations of antibiotics. Indeed, when a non-preferred C-source (lactamide) was added together with sub-inhibitory doses of ciprofloxacin or cefepim, biofilm formation was less pronounced when compared with the antibiotic alone (Figures 13 and 14). In contrast, the addition of a preferred C-source (succinate) resulted in a 6-fold increase in biofilm formation when compared to the antibiotic alone (Figure 13). Previous studies showed that CrcZ indirectly impacts on biofilm formation by competing for Hfq (Pusic *et al*., 2016). Thus, these results corroborate the hypothesis that the induced synthesis of CrcZ could be harnessed to decrease biofilm formation, which in turn seems to increase the efficacy of antibiotics.

In our study, we employed a commonly used microtiter plate-based crystal violet assay to indirectly quantify biofilm formation. Although this assay is a high-throughput method and is widely used for assessing biofilm formation, it is known for its low reliability, which can lead to variable results from experiment to experiment, and even from well to well due to growth and handling. Indeed, we observed same technical variabilities in our experiments (Figures 13 and 14). Therefore, this assay may lead to flawed conclusions and should be used as a screening tool rather than as a stand-alone tool (Kragh *et al*., 2019). Thus, it appears worthwhile in future studies to employ bacterial viability staining in combination with automated confocal laser scanning microscopy (Müsken *et al*., 2010). This method can be used to identify antimicrobial resistance under biofilm growth conditions and to test the impact of novel antimicrobial compounds on bacterial biofilms (Müsken *et al*., 2010). Moreover, it was reported that biofilms grown on biotic surfaces are distinct from biofilms grown on plastic (Anderson *et al*., 2008; Tran *et al*., 2014; Price *et al*., 2015). Price *et al*. (2015) demonstrated that mannitol enhanced antibacterial activity of tobramycin only in biofilms formed on abiotic surfaces but not in biofilms formed on CF airway cells. Therefore, it seems to be of interest to assess the antibiotic susceptibility by adding non-preferred carbon sources to biofilms grown on biotic surfaces. There are several model systems available such as bacterial biofilms formed on the surface of CF-derived airway cells (Price *et al*., 2015), the human skin infection model (Rancan *et al*., 2019) or the murine solid tumor model (Pawar *et al*., 2014).

5. References

Aanaes, K., Rickelt, L. F., Johansen, H. K., von, B. C., Pressler, T., Hoiby, N. & other authors (2011). Decreased mucosal oxygen tension in the maxillary sinuses in patients with cystic fibrosis. *J Cyst Fibros* **10**, 114–120.

Abdou, L., Chou, H.T., Haas, D. & Lu, C.D. (2011). Promoter recognition and activation by the global response regulator CbrB in *Pseudomonas aeruginosa*. *J Bacteriol* **193**(11), 2784-2792. doi: 10.1128/JB.00164-11.

Ahmed, M. N., Porse, A., Sommer, M. O. A., Høiby, N. & Ciofu, O. (2018). Evolution of antibiotic resistance in biofilm and planktonic *Pseudomonas aeruginosa* populations exposed to subinhibitory levels of ciprofloxacin. *Antimicrob Agents Chemother*, **62**: e00320-18. https://doi.org/10.1128/AAC.00320-18.

Aiba, H. (2007). Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr Opin Microbiol* **10**, 134–139.

Alhede, M., Holm Jakobsen, T. & Givskov, M. (2011). Novel and future treatment strategies, p 231–250. *In* Bjarnsholt T, Moser C, Jensen Pø, Høiby N(ed), *Biofilm infections*. Springer, London, United Kingdom.

Allison, K. R., Brynildsen, M. P. & Collins, J. J. (2011). Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* **473**, 216-220.

Anderson, G. G., Moreau-Marquis, S., Stanton, B. A. & O'Toole, G. A. (2008). In vitro analysis of tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived airway epithelial cells. *Infect Immun* **76**(4), 1423–33.

Bagge, N., Schuste, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P. & other authors (2004). *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. *Antimicrob Agents Chemother* **48**, 1175–1187.

Barken, K. B., Pamp, S. J., Yang, L., Gjermansen, M., Bertrand, J. J., Klausen, M., Givskov, Whitchurch, C. B., Engel, J. N. & Tolker-Nielsen, T. (2008). Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* **10** (9), 2331–2343.

Barraud, N., Buson, A., Jarolimek, W. & Rice, S. A. (2013). Mannitol enhances antibiotic sensitivity of persister bacteria in *Pseudomonas aeruginosa* biofilms. *PLoS One* **8**, e84220.

Barroso, R., Garcıa-Mauriño, S. M., Tomas-Gallardo, L., Andujar, E., Perez-Alegre, M., Santero, E. & Canosa, I. (2018). The CbrB Regulon: Promoter dissection reveals novel

insights into the CbrAB expression network in *Pseudomonas putida*. PLoS ONE **13**(12): e0209191. https://doi.org/10.1371/journal.pone.0209191

Basu Roy, A. & Sauer, K. (2014). Diguanylate cyclase NicD-based signaling mechanism of nutrient-induced dispersion by *Pseudomonas aeruginosa*. *Mol Microbiol* **94**, 771–793.

Bernier, S. P., Ha, D. G., Khan, W., Merritt, J. H. & O'Toole G. A. (2011). Modulation of *Pseudomonas aeruginosa* surface-associated group behaviors by individual amino acids through c-di-GMP signaling. *Res Microbiol* **162**, 680–688.

Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sorensen, S. R., Moser, C., Kuhl, M. & other authors (2013). The in vivo biofilm. *Trends Microbiol* **21**, 466–474.

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nature Rev Microbiol* **13**(1), 42-51.

Bowler, L. L., Zhanel, G. G., Ball, T. B. & Saward, L. L. (2012). Mature *Pseudomonas aeruginosa* biofilms prevail compared to young biofilms in the presence of ceftazidime. *Antimicrob Agents Chemother* **56**, 4976–4979.

Brencic, A. & Lory, S. (2009). Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* **72**, 612–632.

Brencic, A., McFarland, K. A., McManus, H. R., Castang, S., Mogno, I., Dove, S. L. & Lory, S. (2009). The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbial* **73**(3), 434-445.

Brennan, R. G. & Link, T. M. (2007). Hfq structure, function and ligand binding. *Curr Opin Microbiol* **10**, 125–133.

Brown, M. & Smith, A. (2003). Antimicrobial agents and biofilms, p 36–55. *In* Wilson M, Devine D (ed), Medical implications of biofilms. Cambridge University Press, New York, NY.

Browne, P., Barret, M., O'Gara, F. & Morrissey, J. P. (2010). Computational prediction of the Crc regulon identifies genus-wide and species-specific targets of catabolite repression control in Pseudomonas bacteria. *BMC Microbiol* **10**, 300

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D. & Buret, A. (1999). The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* **37**, 1771–1776.

Chambonnier, G., Roux, L., Redelberger, D., Fadel, F., Filloux, A., Sivaneson, M., de Bentzmann, S. & Bordi, C. (2016). The hybrid histidine kinase LadS Forms a multicomponent signal transduction system with the GacS/GacA two-component system in *Pseudomonas aeruginosa*. *PLoS Genet* **12**, e1006032.

Ciofu, O., Nielsen, T. T., Jensen, P. O., Wang, H. & Høiby, N. (2015). Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv Drug Deliv Rev* **85**, 7-23.

Ciofu, O., Rojo-Molinero, E., Macia, M. D. & Oliver, A. (2017). Antibiotic treatment of biofilm infections. *APMIS* **125**, 304–319.

Ciofu, O. & Tolker-Nielsen, T. (2019). Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents-how *P. aeruginosa* can escape antibiotics. *Front Microbiol* **10**, 913.

Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.

Davey, M. E., Caiazza, N. C. & O'Toole, G. A. (2003). Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**(3), 1027-36.

de Fernandez, M. T. F., Eoyang, L. & August, J. T. (1968). Factor fraction required for the synthesis of bacteriophage Q beta-RNA. *Nature* **219**, 588–590.

De Groote, G., Verstraeten, V. N., Fauvart, M., Kint, C. I., Verbeeck, A. M., Beullens, S., Cornelis, P. & Michiels, J. (2009). Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. *FEMS Microbiol Lett* **297**, 73–79.

Delihas, N. & Forst, S. (2001). MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. *J Mol Biol* **313**, 1–12.

Deutscher, J. (2008). The mechanisms of carbon catabolite repression in bacteria. *Curr Opin Microbiol* **11**, 87–93.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**(9), 881–890.

Du, D., Wang-Kan, X., Neuberger, A., van Veen, H. W., Pos, K. M., Piddock, L. J. V. & Luisi, B. (2018). Multidrug efflux pumps: structure, function and regulation. *Nat Rev Microbiol* **16**, 523–539.

Ducret, V., Gonzalez, M. R., Scrignari, T. & Perron, K. (2016). OprD repression upon metal treatment requires the RNA chaperone Hfq in *Pseudomonas aeruginosa*. *Genes* **7**, 82-82.

Fair, R. J. & Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspect Med Chem* **6**, 25.

Fernández, L., Breidenstein, E. B. & Hancock, R. E. (2011). Creeping baselines and adaptive resistance to antibiotics. *Drug Resist Updat* **14**(1), 1-21.

Fernandez-Barat, L., Ciofu, O., Kragh, K. N., Pressler, T., Johansen, U., Motos, A. & other authors (2017). Phenotypic shift in *Pseudomonas aeruginosa* populations from cystic fibrosis lungs after 2-week antipseudomonal treatment. *J Cyst Fibros* **16**, 222–229.

Fernández, L. & Hancock, R. E. W. (2012). Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev* **25**(4), 661-681.

Ferrara, S., Carloni, S., Fulco, R., Falcone, M., Macchi, R. & Bertoni, G. (2015). Post-transcriptional regulation of the virulence-associated enzyme AlgC by the σ^{22}-dependent small RNA ErsA of *Pseudomonas aeruginosa*. *Environ Microbiol* **17,** 199-214.

Flemming, H. C. & Wingender, J. (2010). The biofilmmatrix. *Nat Rev Microbiol* **8**(9), 623–633.

Fröhlich, K. S. & Vogel, J. (2009). Activation of gene expression by small RNA. *Curr Opin Microbiol* **12**, 674–682.

Garcia-Maurino, S. M., Perez-Martinez, I., Amador, C. I., Canosa, I. & Santero, E. (2013). Transcriptional activation of the CrcZ and CrcY regulatory RNAs by the CbrB response regulator in *Pseudomonas putida*. *Mol Microbiol*. **89**(1):189–205.

Garneau-Tsodikova, S. & Labby, K. J. (2016). Mechanisms of resistance to aminoglycoside antibiotics: overview and perspectives. *Med Chem Comm* **7**(1), 11-27.

Gilleland, L. B., Gilleland, H. E., Gibson, J. A. & Champlin, F. R. (1989). Adaptive resistance to aminoglycoside antibiotics in *Pseudomonas aeruginosa*. *J Med Microbiol* **29**(1), 41-50.

Gooderham, W. J. & Hancock, R. E. W. (2008). Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* **33**, 279–294.

Goodman, A. L., Merighi, M., Hyodo, M., Ventre, I., Filloux, A. & Lory, S. (2009). Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev* **23**(2), 249-259.

Görke, B. & Stülke, J. (2008). Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* **6**, 613–624.

Govan, J. R. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderiacepacia*. *Microbiol Rev* **60**, 539-74.

Guillier, M., Gottesman, S. & Storz, G. (2006). Modulating the outer membrane with small

RNAs. *Genes Dev* **20**, 2338-2348.

Hammer, B. K. & Bassler, B. L. (2007). Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci U.S.A.* **104**, 11145–11149.

Hansen, A. M. & Kaper, J. B. (2009). Hfq affects the expression of the LEE pathogenicity island in entero- haemorrhagic *Escherichia coli*. *Mol Microbiol* **73**, 446–465.

Hayashi-Nishino, M., Fukushima, A. & Nishino, K. (2012). Impact of Hfq on the intrinsic drug resistance of *Salmonella enterica* Serovar *Typhimurium*. *Front microbiol* **3**(205), 1-5.

Heeb, S., Blumer, C. & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* **184**, 1046–1056.

Hengzhuang, W., Ciofu, O., Yang, L., Wu, H., Song, Z., Oliver, A. & Niels Høibya, N. (2013). High β-Lactamase Levels Change the Pharmacodynamics of β-Lactam Antibiotics in *Pseudomonas aeruginosa* Biofilms. *Antimicrobial Agents and Chemotherapy* **57**(1), 196–204.

Hengzhuang, W., Wu, H., Ciofu, O., Song, Z. & Høiby, N. (2012). In vivo pharmacokinetics/pharmacodynamics of colistin and imipenem in *Pseudomonas aeruginosa* biofilm infection. *Antimicrob Agents Chemother* **56**, 2683–2690.

Hernández-Arranz, S., Sánchez-Hevia, D., Rojo, F. & Moreno, R. (2016). Effect of Crc and Hfq proteins on the transcription, processing, and stability of the *Pseudomonas putida* CrcZ sRNA. *RNA* **22**, 1902-1917.

Heurlier, K., Dénervaud, V., Pessi, G., Reimmann, C. & Haas, D. (2003). Negative control of quorum sensing by RpoN (σ^{54}) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**, 2227–2235.

Hickman, J. W., Tifrea, D. F. & Harwood, C. S. (2005). A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci U.S.A.* **102**, 14422–14427.

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A. & Miller, S. I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436**, 1171–1175.

Høiby, N., Bjarnsholt, T., Moser, C., Bassi, G. L., Coenye, T., Donelli, G. & other authors (2015). ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. *Clin Microbiol Infect* **21** (Suppl. 1), S1–S25.

Holloway, B. W., Krishnapillai, V. & Morgan, A. F. (1979). Chromosomal genetics of *Pseudomonas*. *Microbiol Rev* **43**, 73-102.

Hooper, D. C. & Jacoby, G. A. (2015). Mechanisms of drug resistance: quinolone resistance. *Ann N Y Acad Sci* **1354**, 12–31.

Jenal, U., Reinders, A. & Lori, C. (2017). Cyclic di-GMP: second messenger extraordinaire. *Nat Rev Microbiol* **15**, 271–284.

Jensen, P. O., Kolpen, M., Kragh, K. N. & Kuhl, M. (2017). Microenvironmental characteristics and physiology of biofilms in chronic infections of CF patients are strongly affected by the host immune response. *APMIS* **125**, 276–288.

Kambara, T. K., Ramsey, K. M. & Dove, S. M. (2018). Pervasive Targeting of Nascent Transcripts by Hfq. *Cell Rep* **23**, 1543–1552.

Karatan, E. & Watnick, P. (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* **73**(2), 310–347.

Karlowsky, J. A., Zelenitsky, S. A. & Zhanel, G. G. (1997). Aminoglycoside adaptive resistance. *Pharmacotherapy* **17**(3), 549-555.

Kay, E., Humair, B., Denervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C. & Haas, D. (2006). Two GacA dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 6026–6033.

Kerr, K. G. & Snelling, A. M. (2009). *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *J Hosp Infect* **73**(4), 338-344.

Kim, T., Bak, G., Lee, J. & Kim, K. S. (2015). Systematic analysis of the role of bacterial Hfq-interacting sRNAs in the response to antibiotics. *J Antimicrob Chemother* **70**, 1659–1668.

Kint, G., De Coster, D., Marchal, K., Vanderleyden, J. & De Keersmaecker, S. C. (2010). The small regulatory RNA molecule MicA is involved in *Salmonella enterica* serovar *Typhimurium* biofilm formation. *BMC Microbiol* **10**, 276.

Kirisits, M. J. & Parsek, M. R. (2006). Does *Pseudomonas aeruginosa* use intercellular signalling to build biofilm communities? *Cell Microbiol* **8**, 1841–1849.

Kolpen, M., Hansen, C. R., Bjarnsholt, T., Moser, C., Christensen, L. D., van Gennip, M. & other authors (2010). Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis. *Thorax* **65**, 57–62.

Kominos, S. D., Copeland, C. E., Grosiak, B. & Postic, B. (1972). Introduction of *Pseudomonas aeruginosa* into a hospital via vegetables. *Appl Microbiol* **24**, 567-70.

Kragh, K. N., Alhede, M., Kvich, L. & Bjarnsholt, T. (2019). Into the well-A close look at the complex structures of a microtiter biofilm and the crystal violet assay. *Biofilm* **1**, 100006.

Krieg, N. & Holt, J. (1984). Bergy's manual of determinative bacteriology. The Williams and Wilkins Co, Baltimore.

Kucera, M. & Lysenko, O. (1968). The mechanism of pathogenicity of *Pseudomonas aeruginosa*. VI. Isolation and properties of the proteinases toxic for larvae of the greater wax moth *Galleria mellonella* L. *Folia Microbiol* **13**, 288-94.

Kulesus, R. R., Diaz-Perez, K., Slechta, E. S., Eto, D. S. & Mulvey, M. A. (2008). Impact of the RNA chaperone Hfq on the fitness and virulence potential of uropathogenic *Escherichia coli*. *Infect Immun* **76**, 3019–3026.

Lapouge, K., Schubert, M., Allain, F. H. & Haas, D. (2008). Gac/Rsm signal transduction pathway of gammaproteobacteria: from RNA recognition to regulation of social behaviour. Mol Microbiol **67**, 241–253.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N. & other authors (2013). Antibiotic resistance—the need for global solutions. *Lancet infect Dis* **13**(12), 1057-1098.

Lebeaux, D., Ghigo, J. M. & Beloin, C. (2014). Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol Mol Biol Rev* **78**, 510–543.

Lewis, K. (2010). Persister cells. *Annu Rev Microbiol* **64**, 357-372.

Li, W. & Lu, C. D. (2007). Regulation of carbon and nitrogen utilization by CbrAB and NtrBC two-component systems in *Pseudomonas aeruginosa*. *J Bacteriol* **189**, 5413–5420.

Liao, J. & Sauer, K. (2012). The MerR-like transcriptional regulator BrlR contributes to *Pseudomonas aeruginosa* biofilm tolerance. *J Bacteriol* **194**, 4823-4836.

Linares, J. F., Gustafsson, I., Baquero, F. & Martinez, J. L. (2006). Antibiotics as intermicrobial signalling agents instead of weapons. *Proc Natl Acad Sci U.S.A.* **103**, 19484–19489.

Linares, J. F., Moreno, R., Fajardo, A., Martínez-Solano, L., Escalante, R., Rojo, F. & Martínez, J. L. (2010). The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. *Environ Microbiol* **12**(12), 3196-212.

Link, T. M., Valentin-Hansen, P. & Brennan, R. G. (2009). Structure of Escherichia coli Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci USA* **106,** 19292–19297.

Liu, P. (1952). Utilization of carbohydrates by *Pseudomonas aeruginosa. J Bacteriol* **64**, 773–781.

Liu, H., Wang, Q., Liu, Q., Cao, X., Shi, C. & Zhang, Y. (2011). Roles of Hfq in the stress adaptation and virulence in fish pathogen *Vibrio alginolyticus* and its potential application as a target for live attenuated vaccine. *Appl Microbiol Biotechnol* **91**, 353–364.

Lloyd, M. G., Lundgren, B. R., Hall, C. W., Gagnon, L. B. -P., Mah, T-F., Moffat, J. F. & Nomura, C.T. (2017). Targeting the alternative sigma factor RpoN to combat virulence in *Pseudomonas aeruginosa*. *Scientific Reports* **7**:12615. https://doi.org/10.1038/s41598-017-12667-y.

Macia, M. D., Rojo-Molinero, E. & Oliver, A. (2014). Antimicrobial susceptibility testing in biofilm-growing bacteria. *Clin Microbiol Infec* **20**, 981–990.

Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S. & O'Toole, G. A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* **426**, 306-310.

Mahajan-Miklos, S., Tan, M. W., Rahme, L. G. & Ausubel, F. M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. *Cell* **96**, 47-56.

Malone, J. G., Jaeger, T., Spangler, C., Ritz, D., Spang, A., Arrieumerlou, C., Kaever, V., Landmann, R. & Jenal, U. (2010). YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. *PLoS Pathog* **6**, e1000804

Martínez, L. C. & Vadyvaloo, V. (2014). Mechanisms of post-transcriptional gene regulation in bacterial biofilms. *FRONT CELL INFECT MI* **4**(38), 1-15.

Mawer, S. L. & Greenwood, D. (1978). Specific and non-specific resistance to aminoglycosides in *Escherichia coli*. *J Clin Pathol* **31**, 12–15.

Meylan, S., Porter, C. B. M., Yang, J. H., Belenky, P., Gutierrez, A., Lobritz, M. A. & other authors (2017). Carbon sources tune antibiotic susceptibility in *Pseudomonas aeruginosa* via tricarboxylic acid cycle control. *Cell Chem Biol* **24**, 195–206.

Mikkelsen, H., Sivaneson, M. & Filloux, A. (2011). Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ Microbiol* **13**(7)**,** 1666-1681.

Mikulecky, P. J., Kaw, M. K., Brescia, C. C., Takach, J. C., Sledjeski, D. D. & Feig, A. L. (2004). *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nature Struct Mol Biol* **11**, 1206–1214.

Miller, J. H. (1972). Experiments in molecular genetics. *Cold Spring Harbor Laboratory*, 466.

Moller, T., Franch, T., Hojrup, P., Keene, D. R., Bachinger, H. P., Brennan, R. G. & Valentin-Hansen, P. (**2002).** Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol Cell* **9**, 23–30.

Moll, I., Afonyushkin, T., Vytvytska, O., Kaberdin, V. R. & Bläsi, U. **(2003).** Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA* **9**, 1308–1314.

Monteiro, C., Papenfort, K., Hentrich, K., Ahmad, I., LeGuyon, S., Reimann, R. & other authors (2012). Hfq and Hfq-dependent small RNAs are major contributors to multicellular development in *Salmonella enterica* serovar *Typhimurium*. *RNA Biol* **9**, 489–502.

Moreno, R., Fonseca, P. & Rojo, F. (2012). Two small RNAs, CrcY and CrcZ, act in concert to sequester the Crc global regulator in Pseudomonas putida, modulating catabolite repression. *Mol Microbiol* **83**(1), 24–40.

Morero, N. R., Monti, M. R. & Argarana, C. E. (2011). Effect of Ciprofloxacin Concentration on the Frequency and Nature of Resistant Mutants Selected from *Pseudomonas aeruginosa mut*S and *mut*T Hypermutators. *Antimicrob Agents Chemother* (*American Society for Microbiology)*, **55** (8), 3668–3676. doi:10.1128/AAC.01826-10

Morita, Y., Tomida, J. & Kawamura, Y. (2014). Responses of *Pseudomonas aeruginosa* to antimicrobials. *Front Microbiol* **4**(422), 10.3389/fmicb.2013.00422.

Moscoso, J. A., Jaeger, T., Valentini, M., Hui, K., Jenal, U. & Filloux, A. (2014). The diguanylate cyclase SadC is a central player in Gac/Rsm-mediated biofilm formation in *Pseudomonas aeruginosa*. *J Bacteriol* **196**(23)**,** 4081-4088.

Moscoso, J. A, Mikkelsen, H., Heeb, S., Williams, P. & Filloux, A. (2011). The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretionvia c-di-GMP signalling. *Environ Microbiol* **13,** 3128–3138.

Moskowitz, S. M., Foster, J. M., Emerson, J. & Burns, J. L. (2004). Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *J Clin Microbiol* **42**, 1915–1922.

Müsken, M., Di Fiore, S., Römling, U. & Häussler, S. (2010). A 96-well-plate–based optical method for the quantitative and qualitative evaluation of *Pseudomonas aeruginosa* biofilm formation and its application to susceptibility testing. *Nat Protoc* **5**(8), 1460-1469.

Nikaido, H. (1996). Multidrug efflux pumps of Gram-negative bacteria. *J Bacteriol* **178**: 5853–5859.

Nikulin, A., Stolboushkina, E., Perederina, A., Vassilieva, I., Bläsi, U., Moll, I., Kachalova, G., Yokoyama, S., Vassylyev, D., Garber, M. & Nikonov, S. (2005). Structure of *Pseudomonas aeruginosa* Hfq protein. *Acta Crystallogr D Biol Crystallogr* **61**, 141–146.

Nishijyo, T., Haas, D. & Itoh, Y. (2001). The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. *Mol Microbiol* **40**, 917–931.

Norrander, J., Kempe, T. & Messing, J. **(1983).** Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101-106.

Oglesby-Sherrouse, A. & Vasil, M. (2010). Characterization of a heme-regulated non-coding RNA encoded by the prrF locus of *Pseudomonas aeruginosa*. *PLoS ONE* **5**(4), e9930.

Ornston, L. N. (1971). Regulation of catabolic pathways in *Pseudomonas*. *Bacteriol Rev* **35**, 87-116.

O'Toole, G. A., Gibbs, K. A., Hager, P. W., Phibbs, P. V. Jr. & Kolter, R. (2000). The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol* **182**(2), 425-31.

Palmer, K. L., Aye, L. M. & Whiteley, M. (2007). Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* **189**, 8079–8087.

Pamp, S. J., Gjermansen, M., Johansen, H. K. & Tolker-Nielsen, T. (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. *Mol Microbiol* **68**, 223–240.

Pamp, S. J., Gjermansen, M. & Tolker-Nielsen, T. (2007). The biofilm matrix: a sticky framework, in: S. Kjelleberg and M. Givskov (Eds.). The biofilm mode of life: mechanisms and adaptation, Norfolk, UK.

Parkins, M. D., Ceri, H. & Storey, D. G. (2001). *Pseudomonas aeruginosa* GacA, a factor in multi host virulence, is also essential for biofilm formation. *Mol Microbiol* **40**, 1215–1226.

Pawar, V., Crull, K., Komor, U., Kasnitz, N., Frahm, M., Kocijancic, D. & other authors (2014). Murine solid tumours as a novel model to study bacterial biofilm formation in vivo. *J Intern Med* **276**, 130–139.

Pei, X. Y., Dendooven, T., Sonnleitner, E., Chen, S., Bläsi, U. & Luisi, B. F. (2019). Architectural principles for Hfq/Crc-mediated regulation of gene expression. *eLife*, **8**: e43158. DOI: https://doi.org/10.7554/eLife.43158

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Camara, M., Haas, D. & Williams, P. (2001). The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. *Bacteriol* **183**(22), 6676–6683.

Petrova, O. E., Cherny, K. E. & Sauer, K. (2014). The *Pseudomonas aeruginosa* diguanylate cyclase GcbA, a homolog of *P. fluorescens* GcbA, promotes initial attachment to surfaces, but not biofilm formation, via regulation of motility. *J Bacteriol* **196**, 2827–2841.

Petrova, O. E. & Sauer, K. (2010). The novel two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA *rsmZ* through CafA. *J Bacteriol* **192**, 5275-88.

Poole, K. (2005). Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob agents Chemother* **49**(2), 479-487.

Price, K. E., Orazi, G., Ruoff, K. L., Hebert, W. P., O'Toole, G. A. & Mastoridis, P. (2015). Mannitol does not enhance tobramycin killing of *Pseudomonas aeruginosa* in a cystic fibrosis model system of biofilm formation. *PLoS ONE* **10**(10), e0141192.

Pusic, P., Sonnleitner, E., Krennmayr, B., Heitzinger, D. A.,Wolfinger, M. T., Resch, A. Bläsi, U. (2018). Harnessing metabolic regulation to increase Hfq-dependent antibiotic susceptibility in *Pseudomonas aeruginosa*. *Front Microbiol* **9**, 2709.

Pusic, P., Tata, M., Wolfinger, M. T., Sonnleitner, E., Häussler, S. & Bläsi, U. (2016). Cross-regulation by CrcZ RNA controls anoxic biofilm formation in *Pseudomonas aeruginosa. Sci Rep* **6**, 39621.

Rancan, F., Contardi, M., Jurisch, J., Blume-Peytav, U., Vogt, A., Bayer, I. S. & Schaudinn, C. (2019). Evaluation of drug delivery and efficacy of ciprofloxacin-loaded povidone foils and nanofiber mats in a wound-infection model based on ex vivo Human Skin. *Pharmaceutics* **11**, 527.

Ranieri, M. R., Whitchurch, C. B. & Burrows, L. L. (2018). Mechanisms of biofilm stimulation by subinhibitory concentrations of antimicrobials. *Curr Opin Microbiol* **45**, 164–169.

Rasamiravaka, T., Labtani, Q., Duez, P. & El Jaziri, M. (2015). The Formation of Biofilms by *Pseudomonas aeruginosa*: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms. BMRI 2015: Article ID 759348.

Rojo, F. (2010). Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol Rev* **34**, 658–684.

Rojo, F. & Dinamarca, M. A. (2004). Catabolite repression and physiological control. *Pseudomonas: Virulence and Gene Regulation*, ed Ramos JL (Kluwer Academic/Plenum, New York), Vol 2, pp 365–387.

Römling, U., Galperin, M. Y. & Gomelsky, M. (2013). Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* **77**, 1–52.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).

Sandle, T. **(2014).** Understanding how Pseudomonas aeruginosa infects. *Pharm Microbiol*

https://www.pharmamicroresources.com/2014/12/understanding-how-pseudomonas.html

Sauter, C., Basquin, J. & Suck, D. (2003). Sm-like proteins in Eubacteria: the crystal structure of the Hfq protein from *Escherichia coli*. *Nucleic Acids Res* **31**, 4091–4098.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C. & other authors (2000). Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J Bacteriol* **182**, 1215–1225.

Schumacher, M. A., Pearson, R. F., Moller, T., Valentin-Hansen, P. & Brennan, R. G. (2002). Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J* **21**, 3546–56.

Simm, R., Morr, M., Kader, A., Nimtz, M. & Römling, U. (2004). GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol* **53**, 1123–1134.

Skiada, A., Markogiannakis, A., Plachouras, D. & Daikos, G. L. (2011). Adaptive resistance to cationic compounds in *Pseudomonas aeruginosa*. *Int J Antimicrob agents* **37**(3), 187-193.

Smyth, P.F. & Clarke, P.H. (1975). Catabolite repression of *Pseudomonas aeruginosa* amidase: The effect of carbon source on amidase synthesis. *J Gen Microbiol* **90**, 81–90.

Sonnleitner, E., Abdou, L. & Haas, D. (2009). Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **106**(51), 21866-21871.

Sonnleitner, E. & Bläsi, U. (2014). Regulation of Hfq by the RNA CrcZ in *Pseudomonas aeruginosa* carbon catabolite repression. *PLoS Genet* **10**, e1004440.

Sonnleitner, E., Gonzalez, N., Sorger-Domenigg, T., Heeb, S., Richter, A. S., Backofen, R. & other authors (2011). The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. *Mol Microbiol* **80**(4), 868–85.

Sonnleitner, E., Hagens, S., Rosenau, F., Wilhelm, S., Habel, A., Jäger, K. E. & Bläsi, U. (2003). Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1. *Microb Pathog* **35**(5), 217-228.

Sonnleitner, E., Moll, I. & Bläsi, U. (2002). Functional replacement of the *Escherichia coli*

hfq gene by the homologue of *Pseudomonas aeruginosa*. *Microbiology* **148**, 883–891.

Sonnleitner, E., Prindl, K. & Bläsi, U. (2017). The *Pseudomonas aeruginosa* CrcZ RNA interferes with Hfq-mediated riboregulation. *PLoS ONE* **12**(7), e0180887.

Sonnleitner, E., Romeo, A. & Bläsi, U. (2012). Small regulatory RNAs in *Pseudomonas aeruginosa*. *RNA Biol* **9**, 364-371.

Sonnleitner, E., Schuster, M., Sorger-Domenigg, T., Greenberg, E. P. & Bläsi, U. (2006). Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*. *Mol Microbiol* **59**(5), 1542-1558.

Sonnleitner, E., Wulf, A., Campagne, S., Pei, X. Y., Michael, Wolfinger, M. T., Forlani, G., Prindl, K., Abdou, L., Resch, A., Allain, F. H. T., Luisi, B. F., Urlaub, H. & Bläsi, U. (2018). Interplay between the catabolite repression control protein Crc, Hfq and RNA in Hfq-dependent translational regulation in *Pseudomonas aeruginosa*. *Nucleic Acids Res* **46** (3), 1470–1485.

Sorger-Domenigg, T., Sonnleitner, E., Kaberdin, V. R. & Bläsi, U. (2007). Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY. *Biochem Biophys Res Commun* **352**, 769–773.

Stanek, K. A., Patterson-West, J., Randolph, P. S. & Mura, C. **(2017).** Crystal structure and RNA-binding properties of an Hfq homolog from the deep-branching Aquificae: conservation of the lateral RNA-binding mode. *Acta Cryst* **D73**, 294–315.

Storz, G., Altuvia, S. & Wassarman, K. M. (2005). An abundance of RNA regulators. *Annu Rev Biochem* **74**, 199-217.

Storz, G., Vogel, J. & Wassarman, K. M. (2011). Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Mol Cell* **43**(6): 880–891.

Stougaard, P., Molin, S. & Nordström, K. (1981). RNAs involved in copy-number control and in compatibility of plasmid R1. *Proc Natl Acad Sci* **78**(10), 6008–12.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. & other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**, 959-64.

Sutherland, I. W. (2001). The biofilm matrix—an immobilized but dynamic microbial environment. *Trends Microbiol* **9**(5), 222–227.

Tata, M., Wolfinger, M. T., Amman, F., Roschanski, N., Dötsch, A., Sonnleitner, E. & other authors (2016). RNASeq Based Transcriptional Profiling of Pseudomonas aeruginosa PA14 after Short- and Long-Term Anoxic Cultivation in Synthetic Cystic Fibrosis Sputum Medium. *PLoS One* **11**, e0147811.

Tatterson, L. E., Poschet, J. F., Firoved, A., Skidmore, J. & Deretic, V. (2001). CFTR and *Pseudomonas* infections in cystic fibrosis. *Front Biosci* **6**, D890-7.

Tolker-Nielsen, T. (2015). Biofilm development. *Microbiol Spectr* **3**, MB-0001-2014.

Tomizawa, J., Itoh, T., Selzer, G. & Som, T. (1981). Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc Natl Acad Sci* **78**(3), 1421–5.

Torrens, G., Cabot, G., Ocampo-Sosa, A. A., Conejo, M. C., Zamorano, L., Navarro, F. & other authors (2016). Activity of Ceftazidime-Avibactam against clinical and isogenic laboratory *Pseudomonas aeruginosa* isolates expressing combinations of most relevant beta-lactam resistance mechanisms. *Antimicrob Agents Chemother* **60**, 6407–6410.

Totten, P. A., Lara, J. C., Lory, S. (1990). The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *J Bacteriol* **172**, 389–396.

Tran, C. S., Rangel, S. M., Almblad, H., Kierbel, A., Givskov, M., Tolker-Nielsen, T. & other aurhors (2014). The *Pseudomonas aeruginosa* type III translocon is required for biofilm formation at the epithelial barrier. *PLoS Pathog* **10**(11), e1004479.

Trias, J. & Nikaido, H. (1990). Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **34,** 52-57.

Turnbull, L., Toyofuku, M., Hynen, A. L., Kurosawa, M., Pessi, G., Petty, N. K., Osvath, S. R., Ca´ rcamo-Oyarce, G., Gloag, E. S., Shimoni, R. & other authors (2016). Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat Commun* **7**, 11220.

Ueda, A. & Wood, T. K. (2009). Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog* **5**, e1000483.

Valderrey, A. D., Pozuelo, M. J., Jiménez, P. A., Maciá, M. D., Oliver, A. & Rotger, R. (2010). Chronic colonization by *Pseudomonas aeruginosa* of patients with obstructive lung diseases: cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease. *Diagn Microbiol Infect Dis* **68**(1), 20-27.

Valentini, M. & Filloux, A. (2016). Biofilms and cyclic di-GMP (c-di-GMP) signaling: lessons from *Pseudomonas aeruginosa* and other bacteria. *J Biol Chem* **291**(24)**,** 12547-12555.

Valentini, M., Garcia-Maurino, S. M., Perez-Martinez, I., Santero, E., Canosa, I. & Lapouge, K. (2014). Hierarchical management of carbon sources is regulated similarly by the CbrA/B systems in *Pseudomonas aeruginosa* and *Pseudomonas putida*. *Microbiology* **160**(10)**,** 2243-2252.

Valentin-Hansen, P., Eriksen, M. & Udesen, C. (2004). The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51**, 1525-1533.

Vallet, I., Diggle, S. P., Stacey, R. E., Camara, M., Ventre, I., Lory, S., Lazdunski, A., Williams, P., & Filloux, A. (2004). Biofilm Formation in *Pseudomonas aeruginosa*: Fimbrial cup Gene

Clusters Are Controlled by the Transcriptional Regulator MvaT. *J Bacteriol*., **186**(9), 2880–2890.

Van Delden, C. & Iglewski, B. H. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* **4**(4), 551.

Vogel, J. & Luisi, B.F. (2011). Hfq and its constellation of RNA. *Nat Rev Microbiol* **9**, 578–589.

Wagner, E. G. & Simons, R. W. (1994). Antisense RNA control in bacteria, phages, and plasmids. *Annu Rev Microbiol* **48**, 713-742.

Walters III, M. C., Roe, F., Bugnicourt, A., Franklin, M. J. & Stewart, P. S. (2003). Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* **47**,317-323.

Waters, L.S. & Storz, G. (2009). Regulatory RNAs in bacteria. *Cell* **136**: 615–628.

Wenner, N., Maes, A., Cotado-Sampayo, M. & Lapouge, K. (2013). NrsZ: a novel, processed, nitrogen-dependent small non-coding RNA that regulates *Pseudomonas aeruginosa* PAO1 virulence. *Environ Microbiol* **16**(4), 1053–1068.

Werner, E., Roe, F., Bugnicourt, A., Franklin, M. J., Heydorn, A., Molin, S., Pitts, B. & Stewart, P. S. (2004). Stratified growth in *Pseudomonas aeruginosa* Biofilms. *Appl Environ Microbiol* ***70***, 6188-6196.

Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P.C., Gottesman, S, Ochsner U. A. & other authors (2004). Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc Natl Acad Sci USA* **101**(26), 9792–7.

Wilhelm, S., Gdynia, A., Tielen, P., Rosenau, F. & Jaeger, K. E. (2007). The autotransporter esterase EstA of *Pseudomonas aeruginosa* is required for rhamnolipid production, cell motility, and biofilm formation. *J Bacteriol* **189**(18), 6695-703.

Wilhelm, S., Tommassen, J. & Jaeger, K. E. (1999). A novel lipolytic enzyme located in the outer membrane of *Pseudomonas aeruginosa*. *J Bacteriol* **181**(22), 6977-86.

Winsor, G. L., Griffiths, E. J., Lo, R., Dhillon, B. K., Shay, J. A., & Brinkman, F. S. L. (2016). Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res* **44**, D646-53.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C. & other authors (2002). Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. *J Clin Invest* **109**, 317–325.

Wu, X. G., Duan, H. M., Tian, T., Yao, N., Zhou, H. Y. & Zhang, L. Q. (2010). Effect of the *hfq* gene on 2,4-diacetylphloroglucinol production and the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24. *FEMS Microbiol Lett* **309**, 16–24.

Yahr, T. L. & Wolfgang, M. C. (2006). Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol* **62**, 631–640.

Yamada, J., Yamasaki, S., Hirakawa, H., Hayashi-Nishino, M., Yamaguchi, A. & Nishino, K. (2010). Impact of the RNA chaperone Hfq on multidrug resistance in *Escherichia coli*. *J Antimicrob Chemother* **65**, 853–858.

Ye, R. W., Haas, D., Ka, J.-O., Krishnapillai, V., Zimmermann, A., Baird, C. & Tiedje, J. M. (1995). Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J Bacteriol* **177**(12), 3606-3609.

Yeung, A. T., Bains, M. & Hancock, R. E. (2011). The sensor kinase CbrA is a global regulator that modulates metabolism, virulence, andantibiotic resistance in *Pseudomonas aeruginosa*. *J Bacteriol* **193**, 918–931.

Yu, E. W., Aires, J. R. & Nikaido, H. (2003). AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. *J Bacteriol* **185**, 5657–5664.

Zeng, Q., Mc Nally, R. R. & Sundin, G.W. (2013). Global small RNA chaperone Hfq and regulatory small RNAs are important virulence regulators in *Erwinia amylovora*. *J Bacteriol* **195**, 1706–1717.

Zgurskaya, H. I. & Nikaido, H. (2000). Multidrug resistance mechanisms: drug efflux across two membranes. *Mol Microbiol* **37**, 219–225.

Zhang, L., Chiang, W. C., Gao, Q., Givskov, M., Tolker-Nielsen, T., Yang, L. & Zhang, G. (2012). The catabolite repression control protein Crc plays a role in the development of antimicrobial-tolerant subpopulations in *Pseudomonas aeruginosa* biofilms. *Microbiology* **158**, 3014–3019.

Zhang, Y. F., Han, K., Chandler, C. E., Tjaden, B., Ernst, R. K. & Lory, S. (2017). Probing the sRNA regulatory landscape of *P. aeruginosa*: post-transcriptional control of determinants of pathogenicity and antibiotic susceptibility. *Mol Microbiol* **106**(6), 919-937.

Zhang, L. & Mah, T. F. (2008). Involvement of a novel efflux system in biofilm specific resistance to antibiotics. *J Bacteriol* **190**, 4447-4452.

Zhang, X.X. & Rainey, P.B. (2008). Dual involvement of CbrAB and NtrBC in the regulation of histidine utilization in *Pseudomonas fluorescens* SBW25. *Genetics* **178**,185–195.

Zhang, A., Wassarman, K. M., Ortega, J., Steven A. C & Storz, G. (2002). The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* **9**, 11–22.

6. Abstract

In *Pseudomonas aeruginosa* as well as in several *Pseudomonas* spp., the two-component system (TCS) CbrA/CbrB controls a variety of metabolic and behavioral traits needed for adaptation to changing environmental conditions. The histidine kinase CbrA and the response regulator CbrB constitute a TCS that, together with the Crc system, controls carbon metabolic flow in *Pseudomonas* species, allowing the utilization of preferred carbon sources and establishing a carbon/nitrogen balance. In *P. aeruginosa*, CbrB activates the transcription of the CrcZ RNA. CrcZ is a regulatory RNA involved in carbon catabolite repression (CCR) and acts as a decoy for the Hfq RNA chaperone, and thus antagonizes the function of Hfq/Crc repressive complexes on catabolic genes. The CbrA/CbrB system has several functions besides the control of catabolic pathways. It is also involved in the regulation of swarming, biofilm formation, cytotoxicity and antibiotic resistance.

In this study, the effect of CbrB on *crc*Z gene expression was assessed by Northern-blot analyses using a *cbrB* deletion mutant. In addition, the regulation of *crcZ* expression during anaerobiosis was studied using transcriptional reporter fusions. In addition, the possibility of harnessing CrcZ synthesis to increase antibiotic susceptibility of *P. aeruginosa* early stage biofilms was assessed. Two antibiotics, ciprofloxacin (CIP) cefepime (CEF), were tested in $SCFM_{anox}$ medium in combination with preferred (succinate) and non-preferred (mannitol or lactamide) carbon sources.

Our results verified the predicted CbrB, IHF, Anr, and σ^N consensus binding sites present in the *crcZ* promoter and showed that the corresponding transcription factors are necessary for transcriptional activation of CrcZ during anaerobiosis. Sub-inhibitory concentrations of CIP and CEF, when combined with lactamide, reduced biofilm formation under anoxic conditions. The minimal biofilm eradication concentration (MBEC) for CIP in $SCFM_{anox}$ medium without addition of any C-source was 4 µg/ml; while the MBEC for CEF could not be determined.

Keywords: CrcZ, RNA, Anoxic Biofilm, Antibiotic Susceptibility, *P. aeruginosa*

7. Zusammenfassung

In *Pseudomonas aeruginosa* sowie in anderen *Pseudomonas* spp. kontrolliert das Zwei-Komponenten System (TCS) CbrA/CbrB eine Reihe von metabolischen und verhaltensbezogenen Merkmalen, die für die Anpassung an wechselnde Umweltbedingungen benötigt werden. Die Histidinkinase CbrA und der Response Regulator CbrB bilden ein TCS, das gemeinsam mit dem Crc System den metabolischen Kohlenstofffluss in *Pseudomonas* spp. reguliert, wodurch die Verwendung bevorzugter Kohlenstoffquellen ermöglicht wird und eine Kohlenstoff/Stickstoff Balance etabliert wird. In *P. aeruginosa* aktiviert CbrB die Transkription der CrcZ RNA. CrcZ ist eine regulatorische RNA, die in die Kohlenstoff Katabolit Repression (CCR) involviert ist und dabei als Anagonist für das RNA Chaperon Hfq fungiert. Dadurch inhibiert CrcZ die Funktion des Hfq/Crc Komplexes als translatorischem Repressor von katabolischen Genen. Neben der Katabolitregulation hat das CbrA/CbrB System noch andere Funktionen, z.B. ist das CbrA/CbrB System beteiligt an der Regulation des Schwärmens, der Biofilm Bildung, Zytotoxizität und der Antibiotikaresistenz.

In dieser Studie wurde der Effekt von CbrB auf die *crcZ* Expression durch Northernblots mittels *cbrB* Deletionsmutanten analysiert. Darüber hinaus wurde die Regulation der *crcZ* Expression während der Anaerobiose durch den Einsatz verschiedener Mutanten mit einem Reportergen untersucht. Weiters wurde die Möglichkeit in Betracht gezogen, die CrcZ Synthese auszunutzen um die Antibiotikasensitivität von *P. aeruginosa* in frühen Stadien der Biofilmbildung zu erhöhen. Die beiden Antibiotika Ciprofloxacin (CIP) und Cefepime (CEF) wurden in $SCFM_{anox}$ Medium in Kombination mit bevorzugten (Succinat) und nicht-bevorzugten (Mannit oder Lactamid) Kohlenstoffquellen getestet.

Unsere Ergebnisse verifizieren die vorhergesagten CbrB, IHF, Anr und σ^N Konsensus Bindestellen in der *crcZ* Promotorregion und zeigen, dass diese Faktoren essentiell sind für die transkriptionale Aktivierung von *crcZ* während der Anaerobiose. Sub-inhibitorische Konzentrationen von CIP und CEF in Verbindung mit Lactamid reduzierten die Biofilm Bildung unter anoxischen Bedingungen. Die minimale Eradikationskonzentration (MBEC) für CIP in $SCFM_{anox}$ Medium ohne Zugabe von Kohlenstoffquellen ist 4 µg/ml, während die MBEC für CEF nicht bestimmt werden konnte.

Schlüsselwörter: CrcZ RNA, anoxische Biofilme, Antibiotikasensitivität, *P. aeruginosa*