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Petra Pusic

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To my family:

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1. Zusammenfassung

Pseudomonas aeruginosa ist ein metabolisch vielseitiger, multiresistenter, opportunistischer Krankheitserreger. Die metabolische Flexibilität wird durch ein großes Genom ermöglicht, das für zahlreiche regulatorische Gene, Zweikomponentensysteme, sowie für viele Gene kodiert, die am Transport und Metabolismus verschiedener Energiequellen beteiligt sind. Ein globales Regulationssystem stellt die Katabolitrepression dar, die neben metabolischen Prozessen auch entscheidend für die Modulation virulenzassoziierter Prozesse ist. In dieser Studie wurde die Beteiligung der beiden Hauptakteure der Katabolitrepression, des post-transkriptionellen Regulators Hfq und der Hfq-bindenden RNA CrcZ hinsichtlich der Biofilmbildung und Antibiotikaempfindlichkeit untersucht.

Unter Bedingungen, die den Atemwegen von Mukoviszidose-Patienten ähneln, war Hfq essenziell für die Bildung anoxischer Biofilme. Dies wurde auf die Regulation eines zentralen Schrittes der Denitrifikation durch Hfq zurückgeführt. CrcZ war unter diesen Bedingungen die am häufigsten an Hfq gebundene RNA. Damit reduziert CrcZ indirekt die Bildung anoxischer Biofilme. Unter diesen Bedingungen trägt der anaerobe Transkriptionsregulator Anr zur anoxischen Transkription von *crcZ* bei. Weitere Studien indizierten, dass der Sigmafaktor RpoN an der Expression des *anr* Gens beteiligt ist.

P. aeruginosa hfq Deletionsmutanten zeigten eine erhöhte Empfindlichkeit gegenüber verschiedenen Klassen von Antibiotika. Eine RNAseq-basierte Transkriptomstudie wies darauf hin, dass Hfq die Antibiotikasensitivität moduliert, indem es an der Regulation des Energiestoffwechsel, der Zellwand- und Lipopolysaccharid-(LPS)-Synthese, der Aufnahme und dem Efflux von Antibiotika, sowie der c-di-GMP-Synthese beteiligt ist. Da CrcZ als Antagonist für die Hfq-vermittelte Regulation wirkt, wurde eine Strategie entwickelt, um die kontrollierte Synthese von CrcZ als ein Werkzeug zur (Re-) Sensibilisierung von *P. aeruginosa* gegenüber Antibiotika zu nutzen.

In *P. aeruginosa* dienen die äußeren Membranproteine OprD und OpdP als Eintrittspforte für Carbapenem Antibiotika. Im Rahmen dieser Studien wurde gezeigt, dass Hfq durch zwei unterschiedliche molekulare Mechanismen zur translationalen Repression von *oprD* und *opdP* beiträgt. Die negative Kontrolle von *oprD* mRNA erfolgt durch Riboregulation, wobei Hfq zusammen mit den kleinen regulatorischen RNAs ErsA und Sr0161 die Translation hemmt. Im Gegensatz dazu wird die Translation von *opdP* durch einen regulatorischen Komplex, bestehend aus Hfq und dem Katabolit-Repressionsprotein Crc, unterdrückt.

2. Summary

Pseudomonas aeruginosa is a fast adapting, metabolically versatile, multi-drug resistant, opportunistic pathogen. Its vast genomic and metabolic flexibility can be ascribed to its large genome consisting of numerous regulatory genes, two-component systems, as well as gene functions involved in transport and metabolism of various carbon and energy sources. One global regulatory system represents carbon catabolite repression control (CCR), which, in addition to its main role in the regulation of catabolism, is also crucial for the modulation of virulence-associated processes. In this study, the involvement of the two major players of CCR, the post-transcriptional regulator Hfq and the Hfq-titrating RNA CrcZ was assessed with regard to biofilm formation and antibiotic susceptibility.

Under conditions approximating to the lungs of cystic fibrosis patients infected with *P. aeruginosa*, Hfq was essential for the formation of anoxic biofilms, which was attributed to the regulation of a central step preceding denitrification. CrcZ was found to be the most abundant RNA bound to Hfq under these conditions, and to indirectly impede anoxic biofilm formation. Under these conditions, the anaerobic transcriptional regulator Anr contributes to the anoxic expression of *crcZ*. Further studies implicated the sigma factor RpoN in expression of the *anr* gene.

P. aeruginosa *hfq* deletion mutants showed an increased susceptibility to distinct classes of antibiotics. RNAseq-based transcriptome studies indicated that Hfq modulates antibiotic susceptibility by regulating energy metabolism, cell wall and lipopolysaccharide (LPS) synthesis, uptake and efflux of antibiotics, as well as the c-di-GMP synthesis. As Hfq-mediated regulation is abrogated by CrcZ, a strategy was devised to harness controlled synthesis of CrcZ as a tool to (re)sensitize *P. aeruginosa* towards antibiotics.

In *P. aeruginosa*, the outer membrane proteins OprD and OpdP are known to serve as entry ports for carbapenem antibiotics. Here, we report that Hfq contributes to translational repression of

both, *oprD* and *opdP*, by two distinct molecular mechanisms. Negative control of *oprD* mRNA involves canonical riboregulation, whereby Hfq in conjunction with the small regulatory RNAs ErsA and Sr0161 inhibits translation. At variance, *opdP* translation is repressed by a regulatory complex consisting of Hfq and the catabolite repression protein Crc.

3. Introduction

2.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, metabolically versatile bacterium which can thrive in diverse environmental niches. High environmental adaptability of *P. aeruginosa* can be attributed to its large genome size (~5-7 Mbp) harbouring many regulatory genes, two-component signalling systems, and gene functions involved in the transport and metabolism of various carbon and energy sources, contributing to the overall genomic and metabolic flexibility of the bacterium (Stover *et al.*, 2000; Silby *et al.*, 2011).

P. aeruginosa can utilize more than 70 organic compounds as carbon and/or nitrogen sources (Stanier *et al.*, 1966). In contrast to *Escherichia coli* and *Bacillus subtilis*, *P. aeruginosa* preferentially metabolizes organic acids such as tricarboxylic acid (TCA) cycle intermediates or amino acids (Collier *et al.*, 1996; Palmer *et al.*, 2005). *P. aeruginosa* can grow aerobically, as well as anaerobically in the presence of nitrate or nitrite as terminal electron acceptors (Zumft, 1997; Williams *et al.*, 2007; Schobert and Jahn, 2010; Arai, 2011). During anaerobiosis it can also grow to a limited extent by employing arginine (Vander Wauven *et al.*, 1984; Luthi *et al.*, 1990) and pyruvate fermentation, the latter being important for long-term anaerobic survival (Eschbach *et al.*, 2004). *P. aeruginosa* is a mesophilic bacterium, growing optimally at 37 °C, but can also survive in broad temperature ranges from 4°C to over 42°C (Tsuji *et al.*, 1982). Furthermore, the organism can thrive as free-living planktonic cells and form multicellular biofilms (Costerton *et al.*, 1987; Stoodley *et al.*, 2002).

P. aeruginosa has been isolated from terrestrial and aquatic environments (Green *et al.*, 1974; Marques *et al.*, 1979; Pirnay *et al.*, 2005; Khan *et al.*, 2007), as well as from different plant and animal hosts (Hoadley and McCoy, 1968; Mushin and Ziv, 1973; Green *et al.*, 1974; Tan, 2002). Although being ubiquitously present in nature, the organism is predominantly found in habitats associated with

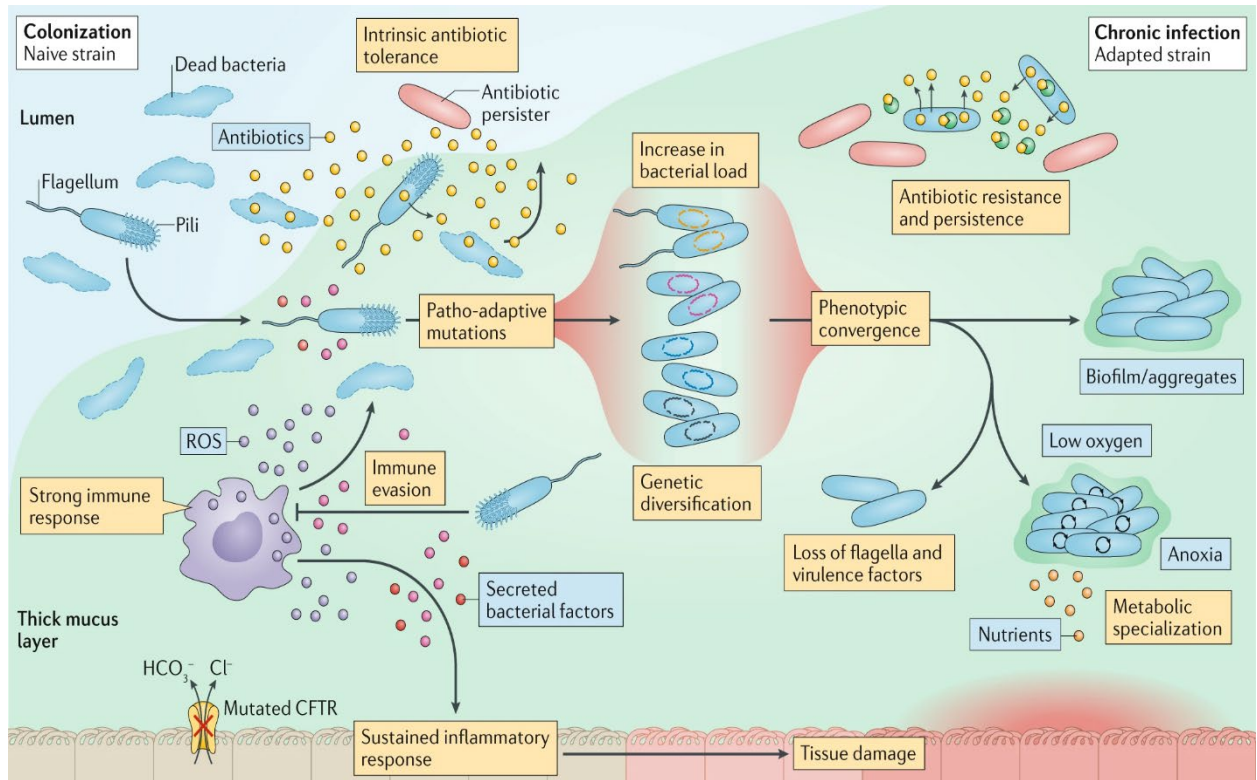


Figure 1. Adaptations of *P. aeruginosa* during chronic infection of cystic fibrosis airway epithelia. During colonization of the cystic fibrosis airways, *P. aeruginosa* is subjected to several selective pressures. Exposure to an initial strong immune response of the host, nutrient and oxygen gradients and high concentrations of antimicrobial agents are the main selective forces, which drive adaptations of a naive strain towards the establishment of a chronic phase of infection (Lyczak *et al.*, 2002; Worlitzsch *et al.*, 2002; Yoon *et al.*, 2002; Folkesson *et al.*, 2012; Marvig *et al.*, 2015; Moradali *et al.*, 2017; Maurice *et al.*, 2018; Rybtke *et al.*, 2020). After the initial die-off phase, a small population of the bacteria is able to escape the immune system and to develop tolerance to antibiotics. Thriving in a constantly changing host environment can lead to the accumulation of patho-adaptive mutations, genetic diversification and independent evolutionary paths in these populations (Folkesson *et al.*, 2012; Marvig *et al.*, 2013; Dötsch *et al.*, 2015; Williams *et al.*, 2015; Moradali *et al.*, 2017). Despite the pronounced genetic heterogeneity (Williams *et al.*, 2015), the adapted populations display phenotypic convergence in the chronic phase of infection (Marvig *et al.*, 2015; La Rosa *et al.*, 2018). Some of the commonly occurring patho-phenotypes are loss of the flagella/motility (Garrett *et al.*, 1999) and diminished synthesis of acute virulence factors (Wu *et al.*, 2004; Smith *et al.*, 2006), metabolic rewiring (Son *et al.*, 2007; Oberhardt *et al.*, 2010; Behrends *et al.*, 2013; Opperman and Shachar-Hill, 2016; La Rosa *et al.*, 2018; Wu *et al.*, 2019), formation of aggregates/biofilms (Bjarnsholt *et al.*, 2009; Hoiby *et al.*, 2010; Mulcahy *et al.*, 2014; Maurice *et al.*, 2018), the acquisition of additional mechanisms of antibiotic resistance and the establishment of persistence (Poole, 2011; Pang *et al.*, 2019). The inflammatory responses of the host and the bacterial activity result in tissue damage and ultimately lead to lung failure (Döring and Worlitzsch, 2000; Hoiby *et al.*, 2010). Adaptive

processes are highlighted in blue boxes, while environmental cues and conditions are shown in brown boxes. CFTR, cystic fibrosis transmembrane conductance regulator; ROS, reactive oxygen species. Figure taken from Rossi *et al.* (2020).

human activity (Crone *et al.*, 2019). *P. aeruginosa* is an opportunistic pathogen that can cause severe acute and chronic infections in immunocompromised individuals, burn patients, and patients suffering from cystic fibrosis (CF) (Bodey *et al.*, 1983; Govan and Deretic, 1996; Manfredi *et al.*, 2000; Kerr and Snelling, 2009). *P. aeruginosa* acute infections are generally characterized by (i) the presence of type IV pili (Bradley, 1980; Comolli *et al.*, 1999) and flagella (Drake and Montie, 1988), which promote twitching and swimming motility (Conrad *et al.*, 2011; Kazmierczak *et al.*, 2015), (ii) expression of the type III secretion functions (T3SS) (Hauser, 2009), and (iii) diminished synthesis of alginate and other exopolysaccharides (Doggett *et al.*, 1964). On the contrary, chronic infections are commonly associated with (i) a diminished or lack of motility (Garrett *et al.*, 1999), (ii) decreased T3SS expression (Wu *et al.*, 2004; Smith *et al.*, 2006), and (iii) increased synthesis of the type VI secretion functions (T6SS) (Sana *et al.*, 2016), alginate (Pedersen, 1992; Wu *et al.*, 2004) and other exopolysaccharides (Ghafoor *et al.*, 2011; Huse *et al.*, 2013), which are important components of biofilms formed in airway epithelia (Lyczak *et al.*, 2002; Bjarnsholt *et al.*, 2009; Hoiby *et al.*, 2010; Folkesson *et al.*, 2012; Moradali *et al.*, 2017; Chen *et al.*, 2020; Harrison *et al.*, 2020; Rossi *et al.*, 2020; Rybtke *et al.*, 2020; Figure 1). Anoxic biofilms represent a preferred mode of growth of *P. aeruginosa* under these conditions (Yoon *et al.*, 2002).

The pathogenic potential of *P. aeruginosa* is based on a combination of several features, including its genomic and metabolic plasticity (Mathee *et al.*, 2008; Behrends *et al.*, 2013), production of a large array of virulence factors (Nadal Jimenez *et al.*, 2012), biofilm formation (Mulcahy *et al.*, 2014; Maurice *et al.*, 2018), as well as intrinsic and acquired resistance towards several classes of antibiotics (Poole, 2011; Pang *et al.*, 2019; Figure 1). These features are controlled by a complex and dynamic regulatory network that enables the bacterium to adapt and thrive in a constantly changing

host environment (Galan-Vasquez *et al.*, 2011; Coggan and Wolfgang, 2012; Balasubramanian *et al.*, 2013; Dötsch *et al.*, 2015; Moradali *et al.*, 2017; Diggle and Whiteley, 2020; Piattelli *et al.*, 2020).

2.2. Carbon and energy metabolism in *P. aeruginosa*

Carbon catabolite repression (CCR) or catabolite repression control (CRC) enables the hierarchical assimilation of carbon sources in bacteria. Under these conditions, bacteria assimilate one preferred carbon source, while the uptake and/or the expression of functions required for the catabolism of others, less energy favourable carbon sources, is inhibited (Collier *et al.*, 1996; Dinamarca *et al.*, 2003; Görke and Stülke, 2008; Rojo, 2010).

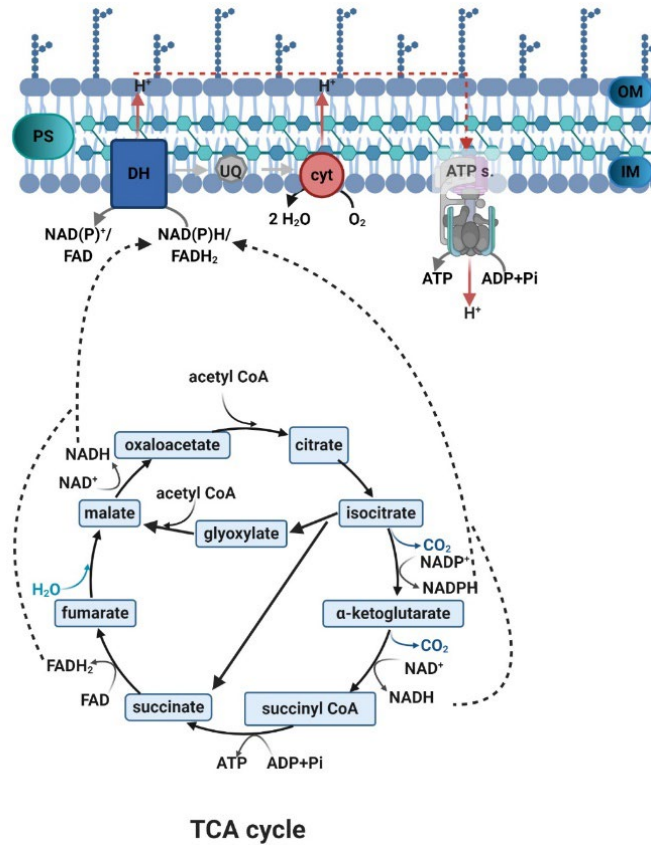
Although glucose is the preferred carbon source for many bacteria, including *E. coli*, *Pseudomonads* preferentially metabolize different organic acids and/or amino acids over monosaccharides (Collier *et al.*, 1996; Palmer *et al.*, 2005, 2007). For example, when both succinate and glucose are present in the medium, the synthesis of enzymes important for glucose catabolism is repressed until succinate is exhausted (Siegel *et al.*, 1977; Collier *et al.*, 1996). Moreover, the expression of genes required for the utilization of other carbohydrates such as fructose, mannitol, gluconate and glycerol, is likewise inhibited by succinate or acetate (Siegel *et al.*, 1977; Roehl and Phibbs, 1982; MacGregor *et al.*, 1991; Collier *et al.*, 1996). Nevertheless, the repressive effect of glucose on amidase genes, as well as genes permitting mannitol or histidine utilization, was observed in *P. aeruginosa* (Brammar and Clarke, 1964; Siegel *et al.*, 1977; Phillips and Mulfinger, 1981; Collier *et al.*, 1996). Catabolite repression of these genes was also observed when the cells were grown in a complete medium, in which amino acids served as the main carbon source (Hester *et al.*, 2000a, 2000b; Rojo, 2010). The same was noted in synthetic CF sputum medium (SCFM) that mimics the nutritional composition of the sputum (mucus) present in the lungs of CF patients (Palmer *et al.*, 2005, 2007). Sputum provides a nutritionally complex substrate that contains several amino acids, glucose

and lactate, which enables long-term colonization of the lungs by *P. aeruginosa* (Ohman and Chakrabarty, 1982; Barth and Pitt, 1995; Thomas *et al.*, 2000; Palmer *et al.*, 2005, 2007; Behrends *et al.*, 2013). In the mucus, amino acids such as phenylalanine, tyrosine, proline, glutamate and aspartate are preferred by *P. aeruginosa* over glucose or lactate (Barth and Pitt, 1995; Palmer *et al.*, 2007, 2010; Son *et al.*, 2007; Behrends *et al.*, 2013). Taken together, there is a hierarchy in the utilization of carbon sources, with organic acids and amino acids being the most preferred, and monohydrates and hydrocarbons being the less preferred carbon sources in *P. aeruginosa* (Valentini *et al.*, 2014).

In most bacteria, the respiration machinery is organized in branched electron transport chains composed of modules (Richardson, 2000). The first module consists of several substrate-specific primary dehydrogenases that oxidize substrates such as NADH, succinate, lactate or other electron donors by transferring the electrons to the ubiquinone pool, which represents the second module. The electrons are then transferred from the reduced ubiquinone (ubiquinol) to the third module comprised of several different terminal oxidases. The terminal oxidases are usually characterized by their different affinity for oxygen, different redox potentials and efficiency as proton pumps (Arai, 2011). *P. aeruginosa* has a branched electron transport chain with five terminal cytochrome oxidases that use oxygen as a final electron acceptor (Williams *et al.*, 2007; Arai *et al.*, 2014; Eichner *et al.*, 2014; Figure 2A).

The composition of the respiratory chains and specifically the synthesis of terminal oxidases is precisely modulated to enable fine-tuning to changing environmental conditions and metabolic needs (Poole and Cook, 2000; Richardson, 2000). For instance, in the presence of high oxygen concentrations, both *P. aeruginosa* and *E. coli* employ a cytochrome *o* ubiquinol oxidase encoded by *cyoABCDE* as the main terminal oxidase. However, when the oxygen levels in the environment decline, this oxidase is substituted by a cytochrome (b)d ubiquinol oxidase encoded by *cydAB* and *cioAB*, respectively, which has a higher affinity for oxygen (Cotter *et al.*, 1990; Cunningham *et al.*, 1997).

A



B

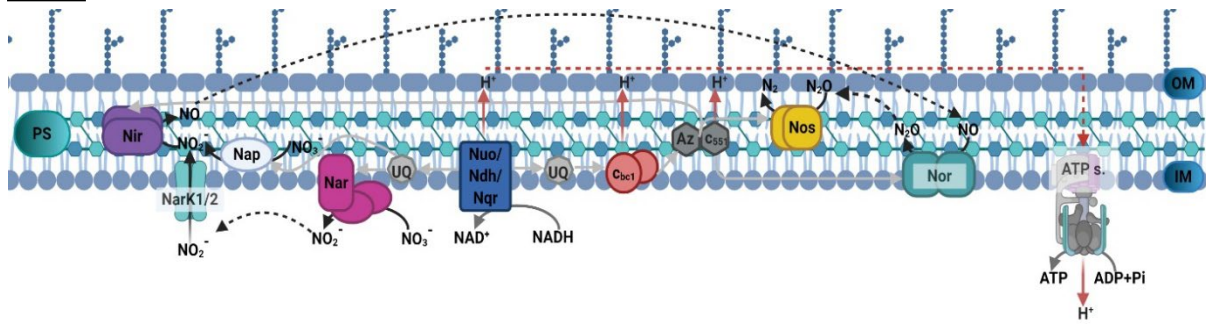


Figure 2. Energy gain of *P. aeruginosa* under aerobic and anaerobic conditions. (A) The tricarboxylic acid (TCA) cycle, aside from ATP synthesis, contributes biosynthetic precursors, as well as reducing agents (NAD(P)H and FADH₂) for downstream reactions. NAD(P)H and FADH₂ are employed by different substrate-specific dehydrogenases (DH; dark blue square) in redox reactions for the generation of a proton (H⁺) gradient (red dashed arrow) across the membrane and in the electron transport chain (light grey arrows). The electrons are then transferred from DH to the ubiquinone pool (grey octagon), which in turn donates them to distinct terminal cytochrome oxidases (cyt; red circle). The aerobic terminal cytochrome oxidases use oxygen (O₂) as a terminal electron receptor and contribute to the proton gradient, which serves as a driving force for ATP production by the ATP synthase (ATPs). The scheme was drawn according to Williams *et al.* (2007), Eichner *et al.* (2014) and

Meylan *et al.* (2017). **(B)** In the absence of oxygen, *P. aeruginosa* is able to use nitrate (NO_3^-) or nitrite (NO_2^-) as alternative electron acceptors in the redox process of denitrification. Key enzymes, electron donors/acceptors and transporters in denitrification are: Nar: inner membrane-bound nitrate reductase, Nap: periplasmic nitrate reductase, Nir: nitrite reductase, Nuo/Ndh/Nqr: NADH-dependent dehydrogenases, UQ: ubiquinone pool, C_{bc1} : cytochrome oxidase bc_1 , Az: azurin, c_{551} : cytochrome c-551, Nor: nitric oxide reductase, Nos: nitrous oxide reductase, ATP s.: ATP synthase; NarK1/2: putative nitrate/nitrite antiporter. The direction of the denitrification reactions is indicated by black arrows. The electron gradient is highlighted by light grey arrows, while the proton gradient is shown by red arrows. The scheme was drawn according to Williams *et al.* (2007) and Borrero-de Acuña *et al.* (2017). OM: outer membrane, IM: inner membrane, PS: periplasmic space.

The transition of *P. aeruginosa* from aerobic to anaerobic growth is controlled by the global transcriptional regulator of anaerobic metabolism, Anr (Ray and Williams, 1997; Trunk *et al.*, 2010; He *et al.*, 2014; Tribelli *et al.*, 2019), an orthologue of the *E. coli* fumarate and nitrate reductase regulator Fnr (Galimand *et al.*, 1991; Sawers, 1991; Zimmermann *et al.*, 1991; Ye *et al.*, 1995). Anr responds to low-oxygen tension *via* iron-sulfur ($[\text{4Fe-4S}]^{2+}$) clusters, which are damaged upon exposure to oxygen (Khoroshilova *et al.*, 1997; Yoon *et al.*, 2007). Under oxygen-limiting conditions, Anr forms active dimers that recognize 5'..TTGATNNNNATCAA..3' motifs in promoter regions (Gamper *et al.*, 1991; Winteler and Haas, 1996). Among others, Anr initiates the synthesis of the nitrate-dependent two-component system NarXL and the nitric oxide-responsive regulator Dnr (Ye *et al.*, 1995; Zumft, 1997). In conjunction with NirQ, these transcription factors regulate the synthesis and exploitation of alternative respiratory branches in the absence of oxygen and in the presence of nitrate or nitrite during the process of denitrification (Arai *et al.*, 1995; Zumft, 1997; Hasegawa *et al.*, 1998; Schreiber *et al.*, 2007; Arai, 2011).

Denitrification represents a multi-step process catalysed by a chain of denitrification enzymes that reduce nitrate to dinitrogen *via* nitrite, nitric oxide and nitrous oxide as alternative electron acceptors (Zumft, 1997; Schobert and Jahn, 2010; Arai, 2011; Borrero-de Acuña *et al.*, 2017; Figure 2B). The first step of denitrification involves the reduction of nitrate to nitrite by two dissimilatory

nitrate reductases (Zumft, 1997); an inner membrane-bound nitrate reductase NarGHI (Carlson *et al.*, 1982) and a periplasmic nitrate reductase NapABC (Carter *et al.*, 1995; Potter and Cole, 1998; Bedzyk *et al.*, 1999). The NarGHI reductase, contributes to the generation of a proton gradient (Zumft, 1997), and is important for anaerobic nitrate respiration of *P. aeruginosa* in the CF mucus (Palmer *et al.*, 2007). In contrast, the NapABC reductase appears not to contribute to the generation of the proton motive force (PMF) and energy during anaerobic denitrification (Van Alst *et al.*, 2009a). However, Nap seems to act as a terminal oxidase, maintaining the redox balance during the transition from aerobic to anaerobic conditions (Davies *et al.*, 1989; Potter and Cole, 1998; Philippot and Højberg, 1999). Moreover, it is deemed to enable a Nar-independent denitrification in aerobic biofilms (Lin *et al.*, 2018b). The generated nitrite effectively binds to heme groups in the electron carriers, and subsequently inhibits the flow of electrons. Due to its toxicity, nitrite is secreted out of the cytoplasm (Rowe *et al.*, 1977). The second step of the denitrification entails a reduction of the toxic nitrite to nitric oxide (NO). This step is facilitated by the periplasmic cytochrome cd1-type reductase NirS. NirS is encoded in the large *nir* operon. The *nirM* and *nirC* genes encode cytochrome c-551 and a periplasmic cytochrome c (Arai *et al.*, 1990; Nordling *et al.*, 1990), respectively, which together with azurin (Arvidsson *et al.*, 1989), function as electron donors for NirS (Kawasaki *et al.*, 1995; Hasegawa *et al.*, 2001). Although NO appears to promote biofilm formation during anaerobiosis (Yoon *et al.*, 2002), it can also be toxic (Yoon *et al.*, 2002) and act as a signal to induce biofilm dispersal under certain conditions (Barraud *et al.*, 2006, 2009). Hence, tight and coordinated regulation of NO synthesis and reduction is of importance for fine-tuning of anoxic biofilm development. The reduction of NO is catalysed by the membrane-bound nitric oxide reductase NorCB, resulting in the generation of the nitrous oxide during the third step in denitrification (Arai *et al.*, 1995; Hendriks *et al.*, 2000; Zumft, 2005). In the final step, the periplasmic nitrous oxide reductase NosZ reduces nitrous oxide to dinitrogen (N₂) (Arai *et al.*, 2003).

In addition to denitrification, *P. aeruginosa* can anaerobically ferment arginine and pyruvate, respectively. Anaerobic conversion of arginine to ornithine is catalyzed by enzymes encoded in the *arcDABC* operon (Vander Wauven *et al.*, 1984; Luthi *et al.*, 1990). The operon encodes members of the arginine deamination pathway, arginine deiminase (ArcA), catabolic ornithine carbamoyltransferase (ArcB), carbamate kinase (ArcC) and an arginine-ornithine antiporter (ArcD). During aerobiosis, pyruvate is converted by pyruvate dehydrogenase (AceE) into acetyl coenzyme A (acetyl-CoA), which is further oxidized in the TCA cycle. However, during anaerobiosis, and in the absence of nitrate, *P. aeruginosa* converts pyruvate to lactate and acetate with the aid of the acetate kinase (AckA), phosphotransacetylase (PtaA) and fermentative D-lactate dehydrogenase (LdhA) (Eschbach *et al.*, 2004; Zlosnik *et al.*, 2006). Anaerobic utilization of arginine supports only limited growth, and *P. aeruginosa* exhibits no growth during pyruvate fermentation (Shoesmith and Sherris, 1960; Eschbach *et al.*, 2004; Schobert and Jahn, 2010). Instead, arginine and pyruvate fermentation enable long-term survival and maintenance of the redox balance in the absence of nitrate or nitrite as terminal electron acceptors (Vander Wauven *et al.*, 1984; Eschbach *et al.*, 2004; Schreiber *et al.*, 2006). The ability to employ alternative respiratory and fermentation pathways permits *P. aeruginosa* to successfully infect and persist in the oxygen-fluctuating environment of the CF lungs (Worlitzsch *et al.*, 2002; Williams *et al.*, 2007; Schobert and Jahn, 2010; Arai, 2011; Behrends *et al.*, 2013).

2.3. Biofilms

Biofilms represent multicellular microbial communities organized in cellular aggregates and/or micro- and macrocolonies encased in a self-produced extracellular matrix (Costerton *et al.*, 1987, 1995; Bjarnsholt *et al.*, 2013; Jensen *et al.*, 2017). The encasing matrix is made of cell-to-cell connecting extracellular polymeric substances (EPS), mostly comprising polysaccharides, DNA, structural proteins and cell debris (Sutherland, 2001; Pamp *et al.*, 2007; Flemming and Wingender, 2010). The exact

composition of the matrix varies depending on the strain, developmental stage of the biofilm and growth conditions (Klausen *et al.*, 2003; Pamp *et al.*, 2007; Lotfy *et al.*, 2018). Depending on the strain, *P. aeruginosa* comprises three main polysaccharides as components of its biofilm matrix: alginate (Pedersen, 1992; Davies *et al.*, 1993; Hoyle *et al.*, 1993; Boyd and Chakrabarty, 1995; Wu *et al.*, 2004), Psl (Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004; Colvin *et al.*, 2012; Irie *et al.*, 2012; Passos da Silva *et al.*, 2019) and Pel (Colvin *et al.*, 2011, 2012; Reichhardt *et al.*, 2020). The biofilm matrix polysaccharides, aside from providing structural integrity to the matrix, enable cell-to-cell interactions and promote biofilm development, as well its dispersion (Sutherland, 2001; Ryder *et al.*, 2007; Ghafoor *et al.*, 2011; Irie *et al.*, 2012; Cherny and Sauer, 2020). In addition, they act as a protective barrier and modulate the host response to *P. aeruginosa* (Huse *et al.*, 2013; Limoli *et al.*, 2015; Harrison *et al.*, 2020; Rybtke *et al.*, 2020). A number of bacterial extracellular structures, such as cup fimbriae, type IV pili and flagella are also involved in cell-to-cell and cell-to-surface connection, and thus can be considered as a part of the biofilm matrix (O'Toole and Kolter, 1998; Vallet *et al.*, 2001; Burrows, 2005, 2012; Toutain *et al.*, 2007; Flemming and Wingender, 2010).

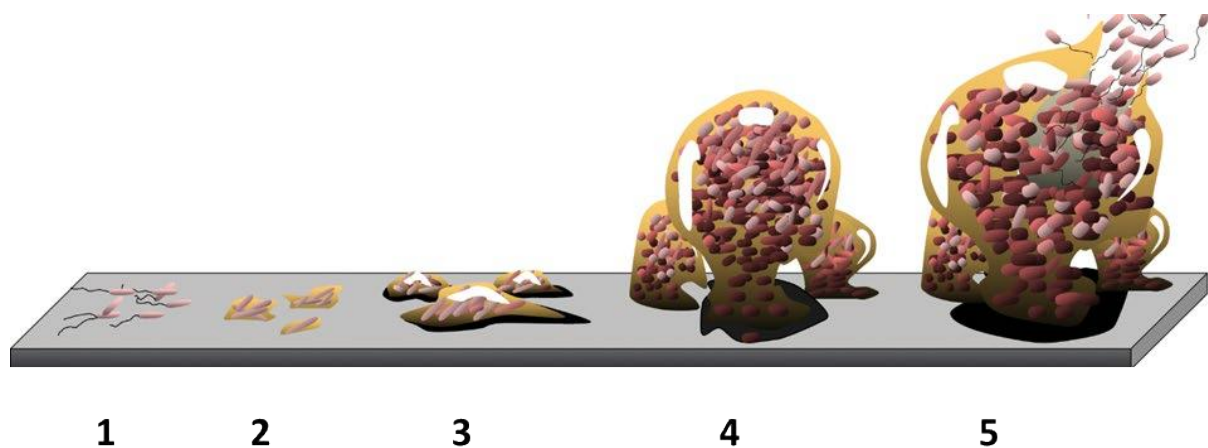


Figure 3. Biofilm development is a complex and dynamic process. Biofilm formation can be divided into five stages: initial reversible attachment (1), irreversible attachment (2), microcolony formation (3), maturation and macrocolony formation (4) and biofilm dispersal (5). Figure adapted from Monroe (2007).

Biofilm formation represents a mode of bacterial growth in response to a variety of environmental cues as it can provide adaptation to (i) nutrient and oxygen deprivation, (ii) interspecies competition, (iii) the host immune response, and (iv) an increased tolerance to antimicrobials (Stoodley *et al.*, 2002; Leid, 2009; Olsen, 2015; Jensen *et al.*, 2017; Ciofu and Tolker-Nielsen, 2019). Biofilm-forming bacterial pathogens are prevalent in chronic infections of humans. For example, *P. aeruginosa* forms microaerophilic and anoxic biofilms in the lungs of patients suffering from CF (Lyczak *et al.*, 2002; Yoon *et al.*, 2002; Hassett *et al.*, 2009; Folkesson *et al.*, 2012; Jensen *et al.*, 2017).

Biofilm development is a complex and dynamic process that can be divided into five stages: 1) initial reversible attachment, 2) irreversible attachment, 3) microcolony formation, 4) maturation and macrocolony formation, and 5) biofilm dispersal (Costerton *et al.*, 1999; Sauer *et al.*, 2002; Monroe, 2007; Figure 3). Biofilm formation is initiated by a reversible attachment of planktonic bacteria to the surface (Figure 3, stage 1). Flagella and type IV pili-mediated binding to the surface and twitching play an important role in this process (O'Toole and Kolter, 1998; Caiazza *et al.*, 2007; Toutain *et al.*, 2007; Burrows, 2012). The attachment is further augmented by generation of the polysaccharide Psl in *P. aeruginosa* strains PAO1 and PA14 (Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004; Ma *et al.*, 2006, 2009) and Pel in strain PAK (Vasseur *et al.*, 2005). In the second stage, the surface attachment becomes irreversible and the bacterial cells become non-motile, forming a monolayer (Sauer *et al.*, 2002; Figure 3, stage 2). Furthermore, the cells produce an extrapolymeric matrix, mainly composed of extracellular DNA (eDNA) (Whitchurch *et al.*, 2002; Gloag *et al.*, 2013; Wang *et al.*, 2015b), whereas polysaccharides and matrix-stabilizing proteins prime biofilm formation as matrix components in later stages (Stoodley *et al.*, 2002; Borlee *et al.*, 2010; Colvin *et al.*, 2011; Passos da Silva *et al.*, 2019; Reichhardt *et al.*, 2020). In the third stage, an intense clonal growth happens, which leads to aggregation and formation of microcolonies (Sriramulu *et al.*, 2005; Figure 3, stage 3). Rhamnolipids seem to be important for this process (Pamp and Tolker-Nielsen, 2007). In the fourth stage, an intense three-dimensional growth and the formation of mature

macrocolonies occurs (Figure 3, stage 4). The formation of mature biofilms is characterized by rhamnolipids-, type IV pili-, flagellum-mediated motility and an eDNA release (Davey *et al.*, 2003; Lequette and Greenberg, 2005; Allesen-Holm *et al.*, 2006; Barken *et al.*, 2008). The spatial structure of macrocolonies depends not only on the strain, but also on the environmental conditions (Pamp *et al.*, 2007; Lotfy *et al.*, 2018). For instance, *P. aeruginosa* forms mushroom-shaped structures in the presence of glucose and flat biofilms in citrate-containing medium (Klausen *et al.*, 2003). Quorum sensing (QS) plays an important role in cell-to-cell communication, especially in mature biofilms during chronic CF lung infection (Davies *et al.*, 1998; Singh *et al.*, 2000). In the last stage, some cells from the mature biofilm start to detach and disperse as free-swimming (planktonic) cells in the environment (Figure 3, stage 5). The detachment and dispersal of cells are highly dependent on the nutritional status and can be triggered by both, the depletion or increase in certain environmental nutrients (Sauer *et al.*, 2004; Gjermansen *et al.*, 2005; Huynh *et al.*, 2012; Kim and Lee, 2016). The dispersal enables bacteria to move to a new niche and initiate a new cycle of biofilm formation.

2.4. Antibiotic resistance

Antimicrobial resistance occurs naturally in the environment and enables bacteria to adapt and resist toxins and antibacterial agents produced by other bacterial species, as well as host immune response (Poole, 2012; Marsland and Gollwitzer, 2014; Surette and Wright, 2017; Rossi *et al.*, 2020; Sommer *et al.*, 2020; Figure 4). Nonetheless, the extensive use of antibiotics in the last decades has led to the emergence of novel antimicrobial resistance mechanisms (Davies and Davies, 2010; Blair *et al.*, 2015). The rise of multidrug-resistant bacteria (bacteria resistant to more than three classes of antibiotics) represents a growing menace to human health (Magiorakos *et al.*, 2012; Tacconelli *et al.*, 2018). Among them, carbapenem-resistant *P. aeruginosa*, together with other antibiotic-resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter*

baumannii, *Pseudomonas aeruginosa*, and *Enterobacter* species) provide a major threat (Tacconelli *et al.*, 2018; De Oliveira *et al.*, 2020). Thus, there is a need for the development of new antimicrobial therapies (Woolhouse *et al.*, 2016; Pang *et al.*, 2019).

P. aeruginosa isolates display resistance to almost all classes of clinically relevant antibiotics, including aminoglycosides, quinolones and beta-lactams (Hansen *et al.*, 2008; Poole, 2011; Yayan *et al.*, 2015). Especially, the *P. aeruginosa* isolates resistant to the last-resort polymyxin and carbapenem antibiotics are increasingly occurring (Falagas *et al.*, 2010; Castanheira *et al.*, 2014; Chalhoub *et al.*, 2016; Abd El-Baky *et al.*, 2020). The major antimicrobial mechanisms of *P. aeruginosa* entail intrinsic, acquired and adaptive resistance (Hancock and Speert, 2000; Lambert, 2002; Lister *et al.*, 2009; Breidenstein *et al.*, 2011; Poole, 2011; Moradali *et al.*, 2017; Pang *et al.*, 2019; Rossi *et al.*, 2020; Figure 4).

Intrinsic resistance, also known as natural insensitivity, of a bacterial species is based on its inherent structural or functional features that permits the respective species to resist the activity of a specific antibiotic (Blair *et al.*, 2015). The high level of intrinsic resistance of *P. aeruginosa* can be attributed to a low outer membrane permeability (Bellido *et al.*, 1992; Hancock and Brinkman, 2002), the synthesis of efflux pumps that actively expel antibiotics out of the cell (Li *et al.*, 1994; Hogan and Kolter, 2002; Sun *et al.*, 2014), and the production of antibiotic-inactivating enzymes, such as β -lactamases and aminoglycoside-modifying enzymes (Okamoto *et al.*, 2001; Poole, 2005; Wolter and Lister, 2013).

On the other hand, *P. aeruginosa* is able to acquire resistance by either horizontal transfer of resistance genes borne on plasmids, transposons, integrons and/or prophages, or as a result of mutational changes that can cause a reduced antibiotic uptake, modifications of antibiotic target(s) or an increased synthesis of efflux pumps and antibiotic-modifying enzymes (Hancock and Speert, 2000; Lambert, 2002; Lister *et al.*, 2009; Breidenstein *et al.*, 2011; Poole, 2011; Pang *et al.*, 2019).

Adaptive resistance is characterized by temporary alterations in gene expression and/or protein levels, which result in phenotypic changes as a response to varying environmental conditions (i.e. nutrient limitation or antibiotic treatment) (López-Maury *et al.*, 2008; Fernández *et al.*, 2011; Fernández and Hancock, 2012; Sandoval-Motta and Aldana, 2016). Whereas intrinsic and acquired

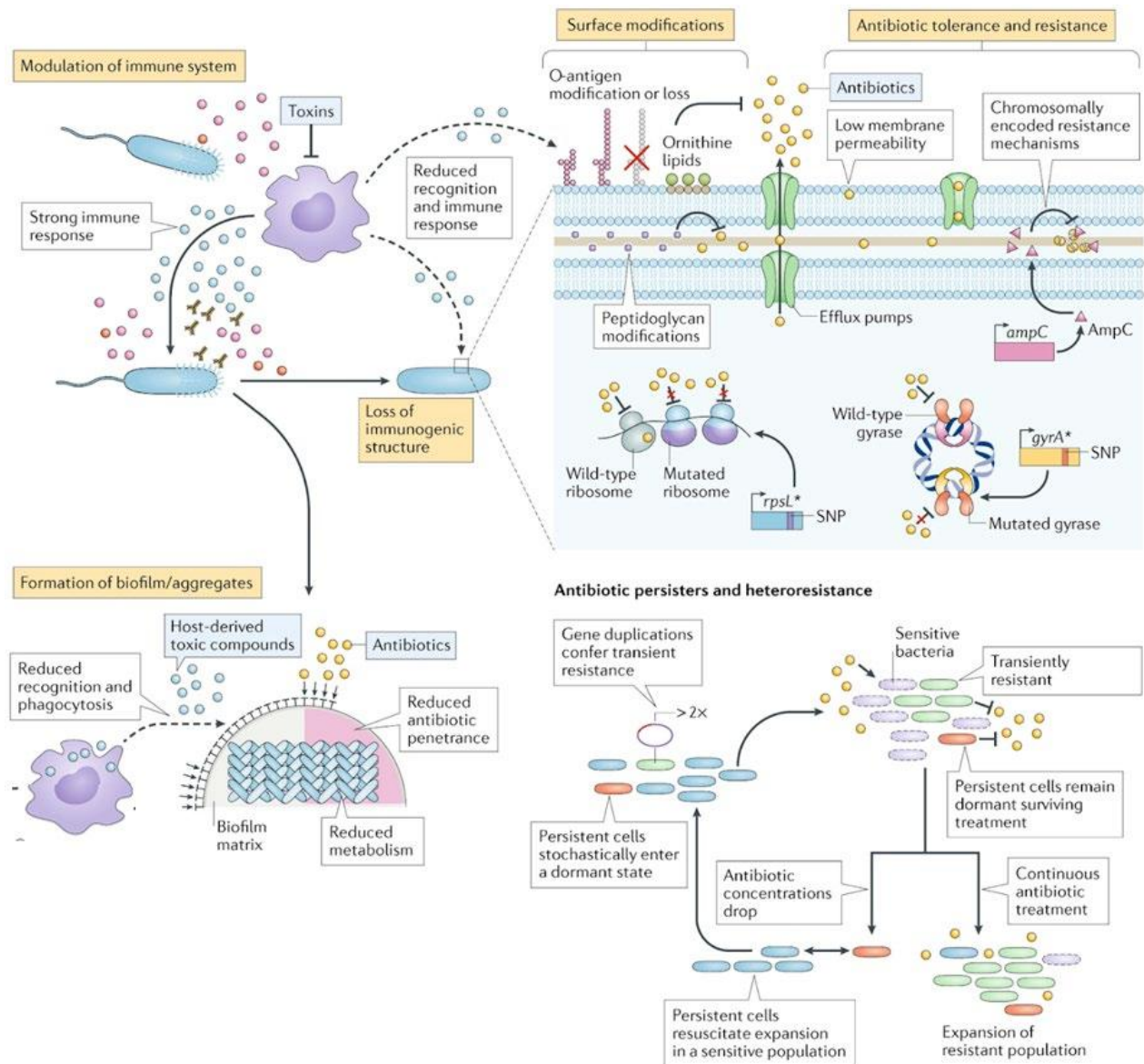


Figure 4. Intrinsic, acquired and adaptive mechanisms of resistance of *P. aeruginosa* to the host immune system and antibiotics. *P. aeruginosa* employs a number of adaptive strategies to evade the immune response of the host, e.g. by the production of extracellular toxins, the loss of immunogenic structures such as flagella and pili and/or by modification of the LPS O-antigen and peptidoglycan as well as through the formation of biofilms/aggregates. The cell surface modifications do

not only reduce immune recognition (Hancock *et al.*, 1983; Ernst *et al.*, 1999; Ernst *et al.*, 2006; Kim *et al.*, 2018), but also promote resistance to certain antibiotics (*e.g.* polymyxins) (Gilleland and Farley, 1982; Miller *et al.*, 2011; Skiada *et al.*, 2011; Fernandez *et al.*, 2013; Lo Sciuto and Imperi, 2018). The thick extracellular biofilm matrix and reduced metabolism of biofilms restrict antibiotic uptake and reduce their effectiveness (Stewart and Costerton, 2001; Drenkard, 2003; Hill *et al.*, 2005; Hoiby *et al.*, 2010; Taylor *et al.*, 2014; Ciofu and Tolker-Nielsen, 2019). Moreover, additional features/mechanisms, including the low outer membrane permeability (Bellido *et al.*, 1992; Hancock and Brinkman, 2002), the synthesis of efflux pumps (Li *et al.*, 1994; Hogan and Kolter, 2002; Sun *et al.*, 2014), and other chromosomally encoded intrinsic and acquired mechanisms of resistance enable the bacterium to thrive under selective antibiotic pressure (Hancock and Speert, 2000; Okamoto *et al.*, 2001; Lambert, 2002; Poole, 2005, 2011; Lister *et al.*, 2009; Breidenstein *et al.*, 2011; Pang *et al.*, 2019). This is further supported by the rise of persistence, heteroresistance (a type of adaptive resistance caused by gene duplication; Nicoloff *et al.*, 2019) and tolerance that lead to the expansion of the resistant population. SNP; single-nucleotide polymorphism. Figure adapted from Rossi *et al.* (2020).

resistance are characterized by an irreversible phenotype and can be transmitted vertically, adaptive resistance is not inherited and usually reverts upon removal of the trigger (Baquero, 2001; Fernández *et al.*, 2011; Fernández and Hancock, 2012; Sandoval-Motta and Aldana, 2016). One of the suggested mechanisms responsible for the emergence of adaptive resistance are epigenetic changes that enable rapid modulation of gene expression (Casadesús and Low, 2006; Adam *et al.*, 2008).

The term adaptive resistance is often mistakenly interchanged with the terms tolerance and persistence (Brauner *et al.*, 2016; Kim and Wood, 2016, 2017; Balaban *et al.*, 2019; Carvalho *et al.*, 2019). However, the main difference is that resistant cells harbour a resistance factor (*e.g.* an efflux pump or an antibiotic-modifying enzyme), and thus are able to grow in the presence of the respective antibiotic by reducing its effectiveness (Brauner *et al.*, 2016). In contrast, tolerant cells are able to survive in the presence of bactericidal antibiotics without carrying a resistance factor, although, they experience a gradual, population-wide decline over time (Brauner *et al.*, 2016; Kim and Wood, 2017; Meylan *et al.*, 2018; Carvalho *et al.*, 2019). On the contrary, persister cells represent a subpopulation of genetically unaltered, metabolically inactive cells that do not grow prior to the antibiotic exposure.

Therefore, persister cells can survive prolonged antibiotic treatment upon an initial die-off of the population (Hobby *et al.*, 1942; Bigger, 1944; Balaban *et al.*, 2013, 2019; Brauner *et al.*, 2016; Carvalho *et al.*, 2019). Resistance, tolerance, and persistence are not mutually exclusive. For instance, tolerance and persistence can promote the emergence of antibiotic resistance (Levin-Reisman *et al.*, 2017; Bakkeren *et al.*, 2019). In addition, resistance and tolerance can act synergistically to promote bacterial survival (Levin-Reisman *et al.*, 2019). In *P. aeruginosa*, the most well-known mechanisms of adaptive antibiotic resistance, tolerance and persistence entail the development of highly-resistant biofilms in the lungs of CF patients (Stewart and Costerton, 2001; Drenkard, 2003; Hill *et al.*, 2005; Hoiby *et al.*, 2010; Taylor *et al.*, 2014; Ciofu and Tolker-Nielsen, 2019), and the generation of multi-drug tolerant persister cells, which are typically associated with a poor prognosis of the CF patients (Drenkard and Ausubel, 2002; Mulcahy *et al.*, 2010; Rossi *et al.*, 2020; Figure 4).

2.5. Metabolic regulation of virulence in *P. aeruginosa*

Nutrient availability and central metabolic networks, which are involved in both carbon and nitrogen metabolism, modulate the expression of virulence genes (Bartell *et al.*, 2017; Panayidou *et al.*, 2020; Perinbam *et al.*, 2020) and impact different virulence traits such as motility (Van Alst *et al.*, 2007; Panayidou *et al.*, 2020), T3SS (Rietsch *et al.*, 2004), QS (Palmer *et al.*, 2007; Zhang *et al.*, 2013; Perinbam *et al.*, 2020), biofilm formation (Van Alst *et al.*, 2007; Perinbam *et al.*, 2020), cytotoxicity (Rietsch *et al.*, 2004) and antibiotic susceptibility (Conrad *et al.*, 1979; Barraud *et al.*, 2013; Meylan *et al.*, 2017). Hence, the metabolic alteration of virulence not only contributes to a better environmental adaptation, but also to the pathogenic potential of *P. aeruginosa* during infection (Palmer and Whiteley, 2015; Panayidou *et al.*, 2020; Rossi *et al.*, 2020; Figure 5).

For instance, the activation of T3SS and T3SS-associated cytotoxicity *in vivo* depends not only on the activity of the anaerobic denitrification enzyme nitrite reductase NirS (Van Alst *et al.*, 2009b),

but also on the expression of the TCA cycle enzymes pyruvate dehydrogenase (Dacheux *et al.*, 2002) and citrate synthase (Rietsch and Mekalanos, 2006). Moreover, histidine and tryptophan catabolism seem to interfere with T3SS synthesis (Rietsch *et al.*, 2004; Shen *et al.*, 2008).

Another example of metabolic fine-tuning of *P. aeruginosa* virulence represents the effect of enhanced bacterial metabolic activity on antibiotic susceptibility. Previous studies have demonstrated that the supplementation of the bacterial growth media with TCA cycle metabolites (*e.g.* succinate, fumarate and citrate) leads to increased bacterial respiration. This in turn leads to an increased PMF, promoting the uptake of PMF-dependent antibiotics, *e.g.* aminoglycosides, and thus to increased aminoglycoside susceptibility (Allison *et al.*, 2011; Meylan *et al.*, 2017). In contrast, diversion of acetyl-coenzyme A through the glyoxylate shunt, a commonly occurring adaptation to the CF environment (Wu *et al.*, 2019; Rossi *et al.*, 2020; Figure 5), represses the TCA cycle, decreases the PMF and leads to enhanced tolerance to aminoglycosides, *e.g.* tobramycin (Meylan *et al.*, 2017). Although several additional studies provided evidence for metabolic regulation of virulence and susceptibility to antibiotics in *P. aeruginosa* (O'Toole *et al.*, 2000; Linares *et al.*, 2010; Yeung *et al.*, 2011, 2014; Sonnleitner *et al.*, 2012b; Reales-Calderón *et al.*, 2015), we are just at the beginning of understanding the underlying molecular mechanisms (Xia *et al.*, 2020a).

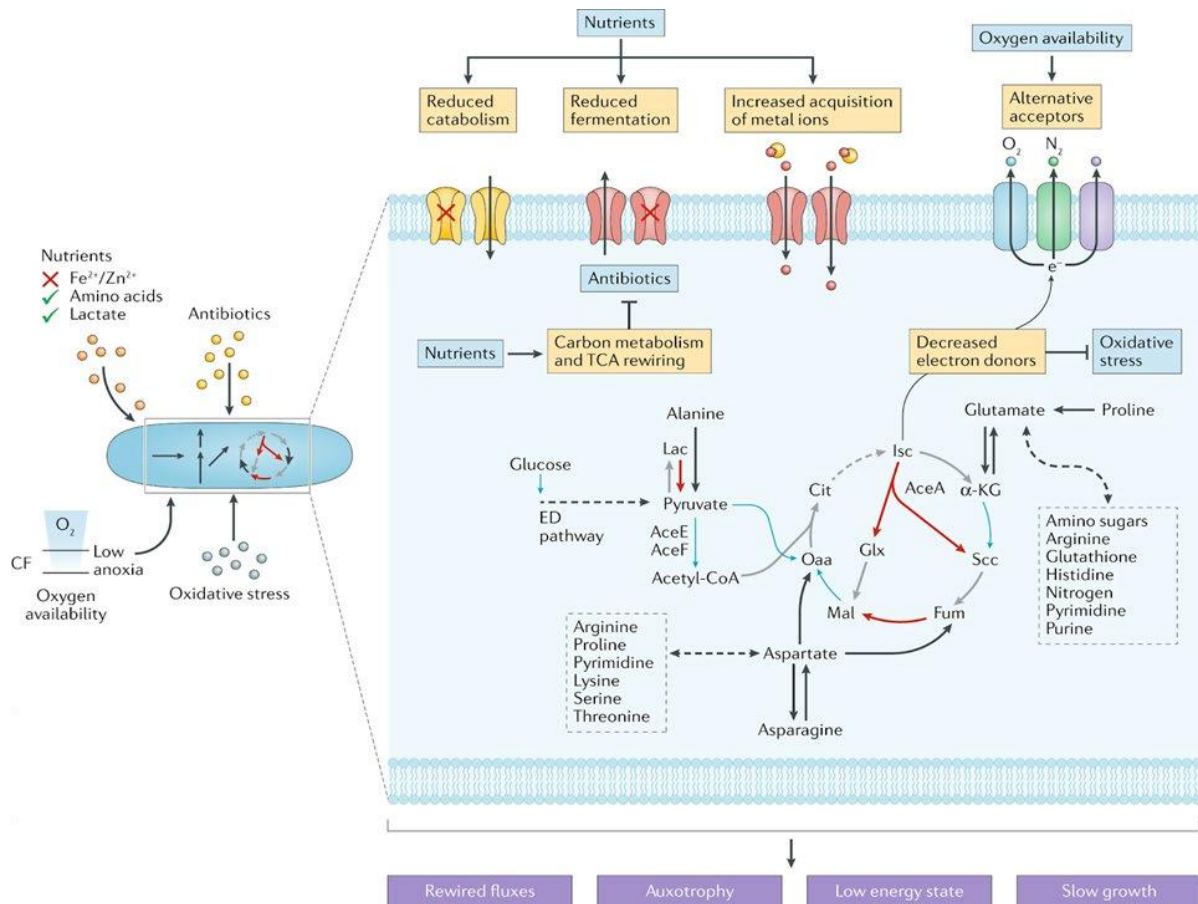


Figure 5. Metabolic alterations of virulence in *P. aeruginosa* during chronic infection in the CF airways. During the adaptation to a long-term infection in the CF lungs, *P. aeruginosa* experiences metabolic rewiring, accompanied with auxotrophy and reduced catabolism of certain abundantly present nutrients (Barth and Pitt, 1995; Son *et al.*, 2007; Behrends *et al.*, 2013; Opperman and Shachar-Hill, 2016; La Rosa *et al.*, 2018). In particular, shifts in central carbon metabolism occur and the reactions connecting the glucose metabolism with the TCA cycle are split (Marvig *et al.*, 2015; Rossi *et al.*, 2018). Instead, the TCA cycle is diverted through the glyoxylate shunt, which is funnelled by the highly abundant lactate and by amino acids (Wu *et al.*, 2019). Although glucose is present at a high concentration, its metabolism is usually decreased in clinical isolates due to their inability to utilize it (La Rosa *et al.*, 2018; Rossi *et al.*, 2018). Reduced glycolysis and the reduction of the lower TCA cycle aid to a decrease in the electron donors in the electron transport chain, which results in a diminished proton motive force (PMF). The decrease in PMF is further assisted by the reduced or completely diminished oxygen availability, which is counteracted by the use of alternative electron acceptors (Zumft, 1997; Son *et al.*, 2007; Williams *et al.*, 2007; Schobert and Jahn, 2010; Arai, 2011). All these metabolic alterations contribute to a low energy state and slow cell growth, and result not only in increased resistance to oxidative stress, but also in diminished effectiveness of certain antibiotics (Meylan *et al.*, 2017; Stokes *et al.*, 2019; Wu *et al.*, 2019). The adaptive processes are indicated by blue boxes, while the environmental signals and conditions are represented by light orange boxes. Increased and decreased metabolic

activity in the CF environment is represented by red and cyan arrows, respectively. Pathways expressed equally both *in vitro* and *in vivo* are indicated by grey arrows. Assimilation pathways are highlighted by black arrows. α -KG, alpha-ketoglutarate; ED, Entner-Dudoroff. Figure taken from Rossi *et al.* (2020).

2.6. Post-transcriptional regulation of metabolism and virulence in *P. aeruginosa*

Tight regulation and coordination of gene expression enable bacteria to robustly modulate their metabolic and virulence traits to increase the fitness and ensure survival. Transcriptional regulation primarily governs individual gene expression, while post-transcriptional control is permitting fast adaptative changes. Gene regulation in *P. aeruginosa* is coordinated by an intricate and interconnected network of numerous sigma factors, transcription factors, two-component systems, as well as post-transcriptional protein regulators and regulatory RNAs (Potvin *et al.*, 2008; Galan-Vasquez *et al.*, 2011; Coggan and Wolfgang, 2012; Balasubramanian *et al.*, 2013; Schulz *et al.*, 2015; Binder *et al.*, 2016; Babitzke *et al.*, 2019; Chevalier *et al.*, 2019; Huang *et al.*, 2019; Arrieta-Ortiz *et al.*, 2020; Piattelli *et al.*, 2020).

2.6.1. RNA-binding proteins

RNA-binding proteins act as post-transcriptional regulators which can affect the maturation, activity and turnover of regulatory small RNAs (sRNAs) in bacteria (Van Assche *et al.*, 2015; Smirnov *et al.*, 2017; Holmqvist and Vogel, 2018; Quendera *et al.*, 2020; Figure 6).

RNA chaperones are a special class of RNA-binding proteins fostering the stability and function of regulatory RNAs (Van Assche *et al.*, 2015; Holmqvist and Vogel, 2018; Woodson *et al.*, 2018; Quendera *et al.*, 2020). In Gram-negative bacteria, the RNA chaperone Hfq and the newly discovered ProQ protein act as RNA matchmakers, aiding in base-pairing between the sRNAs and their target mRNAs (Vogel and Luisi, 2011; Smirnov *et al.*, 2016; Olejniczak and Storz, 2017; Santiago-Frangos and

Woodson, 2018). In contrast, many Gram-positive bacteria lack Hfq and/or ProQ homologs (Bohn *et al.*, 2007; Jousselin *et al.*, 2009; Nielsen *et al.*, 2010; Rochat *et al.*, 2015; Quendera *et al.*, 2020; Ul Haq *et al.*, 2020). A recent study demonstrated that the pleiotropic regulator CsrA, which mainly acts as a direct translational repressor of target mRNAs in *E. coli* and other Gram-negative bacteria, might also be involved in facilitating the interaction between sRNAs and their target mRNAs in *B. subtilis* (Müller *et al.*, 2019).

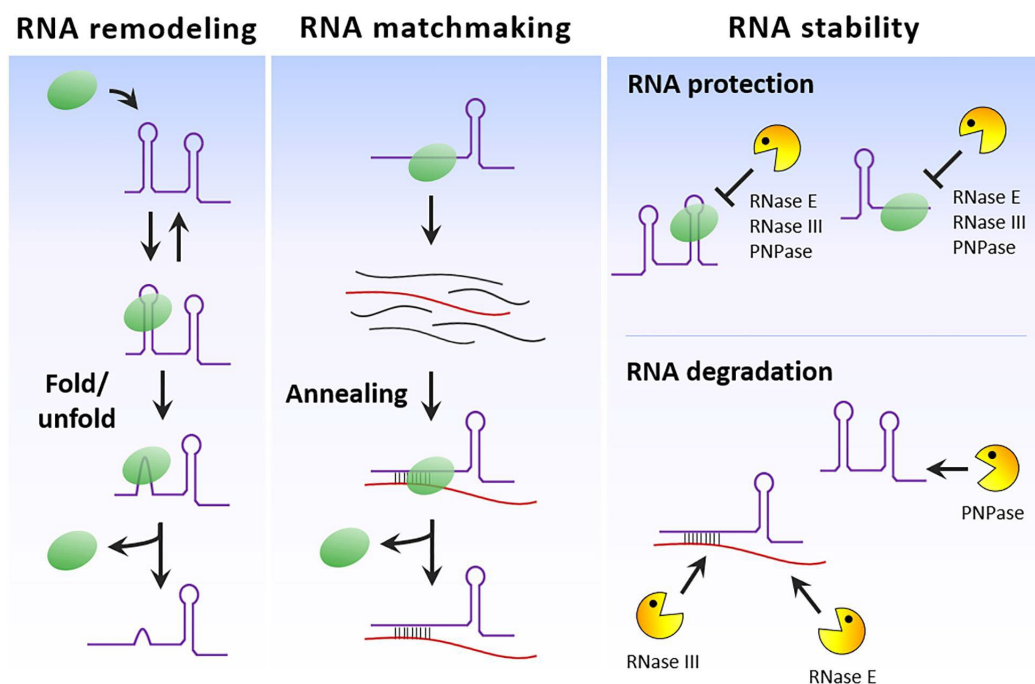


Figure 6. Modes of action of RNA-binding proteins in bacteria. The major RNA chaperones Hfq, ProQ and CsrA (green ovals) are involved in various stages of sRNA (in violet) life-cycles, ranging from sRNA secondary structure remodelling (left panel) to facilitation of sRNA matchmaking with their target mRNAs (in red) (middle panel), and modulation of sRNA stability (upper right panel). Key RNases (RNase E, RNase III and PNPase; yellow pac-men) govern different aspects of sRNA processing and degradation (lower right panel). Figure taken from Quendera *et al.* (2020).

Ribonucleases (RNases) can be regarded as transient RNA-binding proteins with nucleolytic activity, which mediate sRNA processing and turnover, as well as target mRNA decay (Arraiano *et al.*,

2010; Lalaouna *et al.*, 2013; Saramago *et al.*, 2014; Bechhofer and Deutscher, 2019). Key RNases in bacteria include the endonucleases RNase E, RNase Y and RNase III, the endo-/5'-3' exonuclease RNase J and the 3'-5' exonuclease polynucleotide phosphorylase (PNPase) (Durand and Condon, 2018; Mohanty and Kushner, 2018; Bechhofer and Deutscher, 2019).

2.6.2. RNA-binding proteins in *P. aeruginosa*

2.6.2.1. RsmA and RsmN/F

In *P. aeruginosa*, the RNA-binding proteins RsmA (Pessi *et al.*, 2001) and RsmN/F (Marden *et al.*, 2013; Morris *et al.*, 2013) represent two orthologs of the well-studied carbon storage regulator CsrA, which acts primarily as a direct translational repressor and a pleiotropic regulator of various processes in *E. coli* (Romeo and Gong, 1993; Romeo *et al.*, 1993; Sabnis *et al.*, 1995; Potts *et al.*, 2017; Romeo and Babitzke, 2018; Sobrero and Valverde, 2020). The Rsm proteins serve as post-transcriptional regulators of many target genes which are involved in the transition between acute and chronic virulence phenotypes (Burrowes *et al.*, 2006; Lapouge *et al.*, 2008; Mulcahy *et al.*, 2008; Brencic and Lory, 2009; Marden *et al.*, 2013; Romero *et al.*, 2018; Gebhardt *et al.*, 2020; Chihara *et al.*, 2021; Figure 7). This regulation is alleviated in the presence of Rsm-binding RNAs which sequester and titrate the Rsm proteins from their target mRNAs, and thereby promote chronic infection traits. The Rsm RNAs were also reported to be under indirect positive control of Rsm proteins, indicating the complexity of the regulatory network (Chihara *et al.*, 2021; see Figure 20 and below for details).

RsmA and RsmN/F share a similar tertiary homodimeric structure with two identical RNA-binding sites on either side of the dimer, which is characteristic for the CsrA protein family (Rife *et al.*, 2005; Marden *et al.*, 2013; Morris *et al.*, 2013; Sobrero and Valverde, 2020). However, Rsm proteins differ in their secondary structure (Figure 8). RsmA displays a canonical secondary structure common

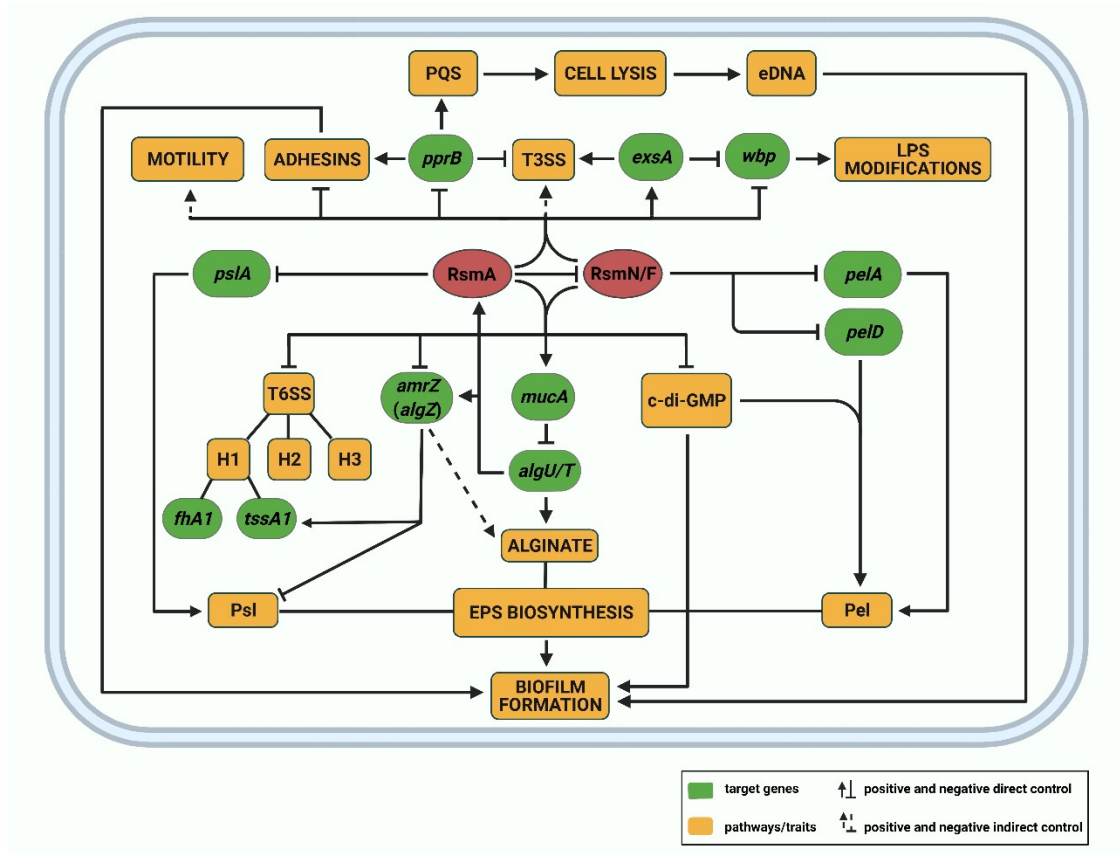


Figure 7. The post-transcriptional regulators RsmA and RsmN/F coordinate the transition between acute and chronic infections. RsmA and RsmN/F proteins (in red) mainly act in concert as direct (straight lines) or indirect (dashed lines) translational repressors or activators of different target mRNAs (in green) and pathways (in yellow), which contribute to the shift between the acute (planktonic) and the chronic (sessile, biofilm) phenotype. The shift to the chronic infection phenotype is mediated by the Rsm RNAs, which act as a decoy for Rsm proteins (see Figure 20 and below for details). RsmN/F protein synthesis is negatively controlled by RsmA at the translational level. ↓: positive control; ⊥: negative control. The scheme was drawn according to Romero *et al.* (2018), Gebhardt *et al.* (2020) and Chihara *et al.* (2021).

for other CsrA proteins, which consists of five highly conserved successive β -strands (β 1- β 5) and a C-terminal α -helix (Gutierrez *et al.*, 2005; Rife *et al.*, 2005; Heeb *et al.*, 2006; Mercante *et al.*, 2006; Figure 8A, C, F). In contrast, in RsmN/F the α -helix is inserted between the β 2- and β 3-strand (Marden *et al.*, 2013; Morris *et al.*, 2013; Figure 8B, D, F). While the C-terminally positioned α -helix in RsmA appears not to be crucial for maintenance of the dimer, in RsmN/F the internally positioned α -helix is

Rsm proteins can negatively regulate translation of their target mRNAs through binding to stem-loop structures that contain GGA-rich motifs in their 5' *untranslated regions* (UTR), which usually impacts their stability and promotes their turnover (Dubey *et al.*, 2005; Lapouge *et al.*, 2007, 2008; Schubert *et al.*, 2007; Marden *et al.*, 2013; Morris *et al.*, 2013; Schulmeyer *et al.*, 2016; Romero *et al.*, 2018; Chihara *et al.*, 2021).

In addition, they can also act as indirect activators and repressors of a large subset of genes by altering the expression of key regulators (Burrowes *et al.*, 2006; Brenic and Lory, 2009; Romero *et al.*, 2018). Furthermore, Rsm protein binding can result in the stabilization of transcripts, and thereby promote their translation (Heurlier *et al.*, 2004; Frangipani *et al.*, 2014; Chihara *et al.*, 2021). The “activity” of Rsm proteins is controlled by the Rsm-binding RNAs, which act by titration. Their mechanism of action will be discussed below (Heurlier *et al.*, 2004; Kay *et al.*, 2006; Janssen *et al.*, 2018a, 2018b; Romeo and Babitzke, 2018; Chihara *et al.*, 2021).

RsmA and RsmN/F act in concert and have overlapping, as well as unique regulatory roles (Marden *et al.*, 2013; Janssen *et al.*, 2018b; Romero *et al.*, 2018; Figure 7). Although the absence of RsmN/F has little or no impact on the expression of RsmA-regulated genes, the deletion of both, *rsmA* and *rsmN/F*, causes additional phenotypes when compared with a single *rsmA* deletion (Marden *et al.*, 2013; Janssen *et al.*, 2018b). In addition, RsmN/F and RsmA exhibit distinct binding affinities for some of their RNA targets (Marden *et al.*, 2013; Schulmeyer *et al.*, 2016; Janssen *et al.*, 2018a, 2018b; Chihara *et al.*, 2021). RsmN/F binds only to targets that contain at least two Rsm consensus sequences, while RsmA requires only a single GGA-motif for efficient interactions (Schulmeyer *et al.*, 2016; Chihara *et al.*, 2021). Moreover, RsmA functions as a direct translational repressor of RsmN/F (Marden *et al.*, 2013; Figure 7). Thus, RsmN/F seems to play an auxiliary role in the Rsm hierarchical regulatory cascade in *P. aeruginosa* (Figure 7).

2.6.2.2. Hfq

Hfq protein was first discovered as a host factor essential for replication of bacteriophage Q β in *E. coli* (Franze de Fernandez *et al.*, 1968). Hfq belongs to the Sm/Lsm family of RNA-binding proteins, ubiquitously present in eukaryotes, as well as many eubacteria and archaea (Sun *et al.*, 2002; Scofield and Lynch, 2008). Hfq is a pleiotropic regulator of many cellular processes related to virulence, metabolism and stress-responses in bacteria (Chao and Vogel, 2010). It is best known for its role in post-transcriptional regulation. As an RNA matchmaker/chaperone it facilitates the interaction between *trans*-encoded sRNAs and their target mRNAs in Gram-negative bacteria (Møller *et al.*, 2002; Ribeiro *et al.*, 2012; Weichenrieder, 2014; Updegrove *et al.*, 2016; Figure 9). Hfq primarily accelerates

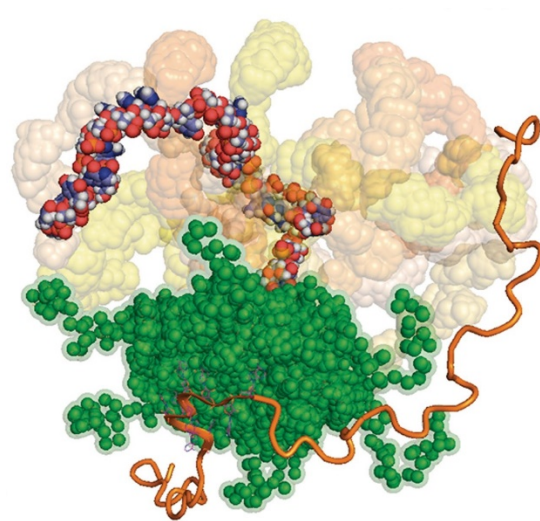


Figure 9. Model for the *E. coli* Hfq RNA matchmaker/chaperone function in riboregulation. *E. coli* Hfq (in green) serves as a platform for transient binding of interacting RNA ligands to either side of the homo-hexameric ring. In this model, the DsrA sRNA (full-atom model, in Corey-Pauling-Koltun (CPK) color scheme) interacts *via* its polyU-rich stretches with the proximal surface of the Hfq hexamer, while the target mRNA (in orange) associates through its polyA-rich motif (flat cartoon view) with the distal surface of Hfq. Hfq thereby increases the local concentration of the interacting RNAs and enables their unfolding and restructuring. The conformational flexibility of the RNA ligands leads to large space coverage, which seems to be further promoted by Hfq and in turn results in stochastic base-pairing between sRNA and mRNA. Figure taken from Ribeiro *et al.* (2012).

annealing of the sRNA to its target (Møller *et al.*, 2002; Zhang *et al.*, 2002; Lease and Woodson, 2004; Rajkowitsch and Schroeder, 2007; Soper and Woodson, 2008; Fender *et al.*, 2010), followed by either mRNA stabilization or decay (Massé *et al.*, 2003; Moll *et al.*, 2003a; Schu *et al.*, 2015).

Hfq binds transiently to sRNAs and mRNAs and its annealing activity was found to occur both, with and without unfolding of target RNAs (Lease *et al.*, 1998; Brescia *et al.*, 2003; Moll *et al.*, 2003b; Geissmann and Touati, 2004; Arluison *et al.*, 2007; Henderson *et al.*, 2013; Peng *et al.*, 2014; Hoekzema *et al.*, 2019). Hfq is further deemed to increase the local concentration of a sRNA and its target mRNA by bringing them into close proximity for base-pairing (Møller *et al.*, 2002; Zhang *et al.*, 2002; Storz *et al.*, 2004; Vogel and Luisi, 2011; Ribeiro *et al.*, 2012; Updegrove *et al.*, 2016; Santiago-Frangos and Woodson, 2018; Woodson *et al.*, 2018; Figure 9). Hfq does not require ATP hydrolysis for its RNA chaperone activity (Rajkowitsch and Schroeder, 2007; Hämmerle *et al.*, 2012). The Hfq chaperone appears to dissociate from sRNA-mRNA duplexes after RNA-RNA annealing due to lower affinity for the sRNA-mRNA duplex when compared with the free RNAs (Fei *et al.*, 2015; Updegrove *et al.*, 2016).

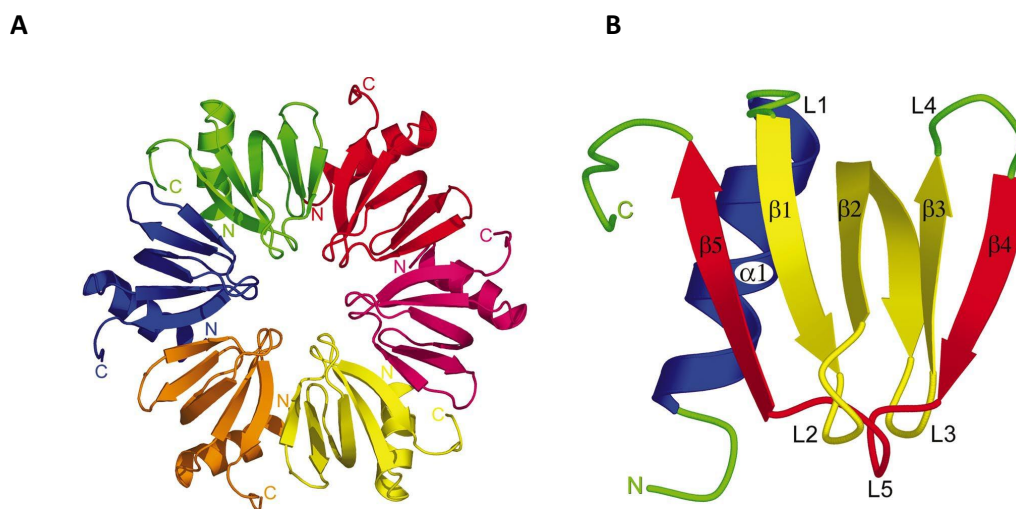


Figure 10. Structure of the *P. aeruginosa* Hfq protein. (A) Structure of the Hfq hexamer with each protomer (monomer, subunit) represented by a different colour. Each subunit is composed of one N-terminal α -helix and five anti-parallel β -strands. (B) Ribbon diagram of the Hfq protomer with the N-terminal α -helix in blue, and the conserved Sm1 and Sm2 motifs highlighted in yellow and red, respectively. The N- and C-termini are indicated with N and C, respectively. Figure taken from Nikulin *et al.* (2005).

Active cycling of competing free RNAs on Hfq may further affect turnover of sRNA-mRNA complexes (Fender *et al.*, 2010; Hwang *et al.*, 2011; Wagner, 2013; Chihara *et al.*, 2019).

The Hfq protomer is composed of two N-terminal structural motifs (Sm1 and Sm2) and a variable intrinsically disordered C-terminal tail. The protein forms a homo-hexameric ring structure (Møller *et al.*, 2002; Schumacher *et al.*, 2002; Sauter *et al.*, 2003; Nikulin *et al.*, 2005; Sauer, 2013; Figure 10) with several distinct RNA binding surfaces (Schumacher *et al.*, 2002; Mikulecky *et al.*, 2004; Link *et al.*, 2009; Vogel and Luisi, 2011; Sauer *et al.*, 2012; Panja *et al.*, 2013; Robinson *et al.*, 2014; Weichenrieder, 2014; Santiago-Frangos and Woodson, 2018; Figures 11-13).

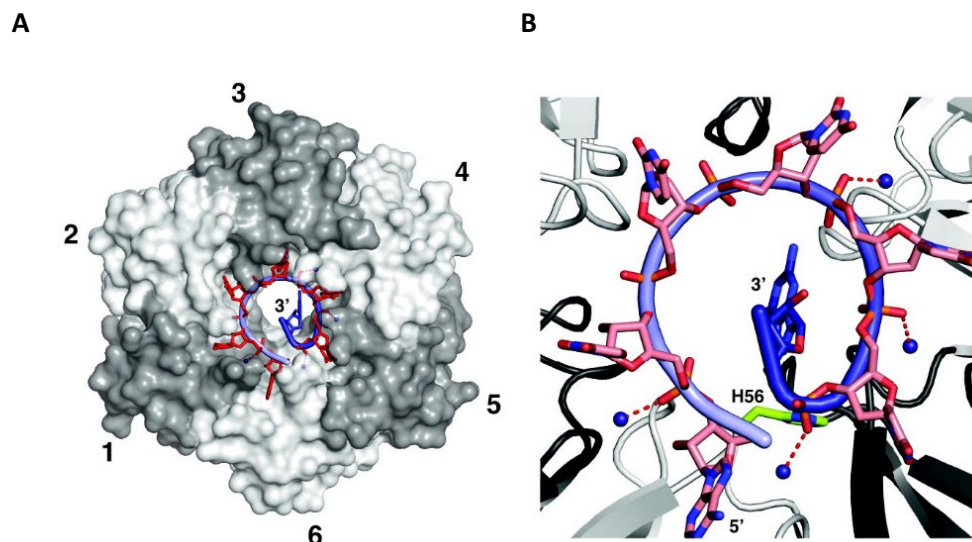


Figure 11. Proximal binding surface of *Staphylococcus aureus* Hfq in complex with an AU₅G-oligonucleotide. (A) Small regulatory RNAs might associate with the Hfq protein *via* their AU₅G-rich sequences by forming a ring around the inner rim of the Hfq proximal binding site (Schumacher *et al.*, 2002). The Hfq protein is shown by a transparent surface model in which each protomer is coloured in alternating white and grey and numbered clockwise. The AU₅G-oligonucleotide is presented as sticks with backbone cartoon coloured in gradient light to dark blue highlighting the 5'-3' direction. **(B)** Enlargement of the inner rim of the Hfq proximal face which is composed of six-nucleotide binding pockets. The pockets are occupied by the first six nucleotides of the AU₅G oligonucleotide, whereas the 3'-terminal guanine is expelled. Hfq protein is presented with a ribbon model, while the oligonucleotide is shown as in **(A)**. The interacting nucleotides are highlighted in red and the expelled guanine is in dark blue. Figure taken from Weichenrieder (2014).

Many sRNAs interact with the proximal binding site of Hfq through either AU₅G-rich sequences (Møller *et al.*, 2002; Schumacher *et al.*, 2002; Mikulecky *et al.*, 2004; Ribeiro *et al.*, 2012) or through the polyU-tails of their rho-independent terminators (Otaka *et al.*, 2011; Sauer and Weichenrieder, 2011; Ishikawa *et al.*, 2012). The interacting sRNAs usually form a ring on the inner rim of the Hfq proximal binding site containing six-nucleotide binding pockets. Each binding pocket binds to a single nucleotide of the interacting RNA (Schumacher *et al.*, 2002; Ribeiro *et al.*, 2012; Weichenrieder, 2014; Figures 9 and 11).

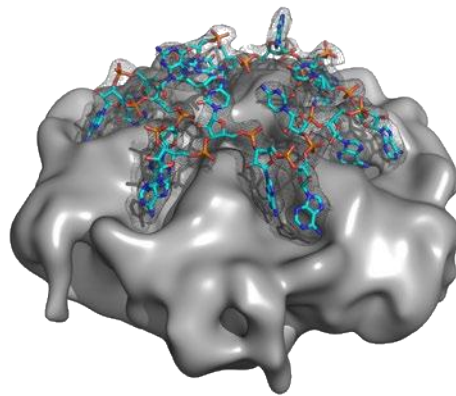


Figure 12. Distal binding surface of *P. aeruginosa* Hfq in complex with *amiE*_{6ARN} RNA. The six ARN-triplets of *amiE* mRNA (stick diagram, in blue and red) are associated with the six tripartite pockets on the Hfq distal binding site and form an “A-R-N” crown (in red). The tripartite pockets consist of A and R-sites which are interacting with an adenine (A) and a purine (R) in the RNA, respectively, while the N-site represents an RNA entry/exit site which does not show any nucleotide preference. Hfq protein (grey) is represented by a surface model. Figure taken from Pei *et al.* (2019).

In contrast, mRNAs can associate with the six tripartite binding pockets located opposite to the proximal site, on the nominal distal binding surface of Hfq (Link *et al.*, 2009; Ribeiro *et al.*, 2012; Murina *et al.*, 2013; Robinson *et al.*, 2014; Sonnleitner and Bläsi, 2014; Pei *et al.*, 2019; Figures 9 and 12) *via* A-rich stretches. The A-rich motifs usually contain ARN triplets (where A is an adenine, R is any purine nucleotide and N is any nucleotide) in *E. coli* and other Gram-negative bacteria (Link *et al.*, 2009), and AN duplets in *B. subtilis* and *S. aureus* (Horstmann *et al.*, 2012; Someya *et al.*, 2012). The

ARN triplets are often located in the vicinity of the *ribosomal binding site* (rbs) of the interacting mRNA and each triplet can bind to one tripartite pocket of the protomer on the Hfq distal site, forming an “A-R-N” crown (Soper and Woodson, 2008; Link *et al.*, 2009; Ribeiro *et al.*, 2012; Robinson *et al.*, 2014; Sonnleitner and Bläsi, 2014; Pei *et al.*, 2019; Figures 9 and 12). Although the A-rich stretches are predominantly found in mRNAs, several studies have shown that some regulatory RNAs also contain A-rich stretches that enable interaction with the distal surface of Hfq (Zhang *et al.*, 2013; Sonnleitner and Bläsi, 2014; Schu *et al.*, 2015; Wang *et al.*, 2015a).

A recent report from Chihara *et al.* (2019) confirmed the preference of *P. aeruginosa* Hfq for AAN triplets in the 5′UTRs, but also for polyU-tails in the 3′UTRs of the target mRNAs. However, in the case of sRNAs, *P. aeruginosa* Hfq seems to favour the association with AAN triplets located in their central region; although, for some sRNAs, the interaction with the GC-rich stem-loop structures of their rho-independent terminators was also observed (Chihara *et al.*, 2019).

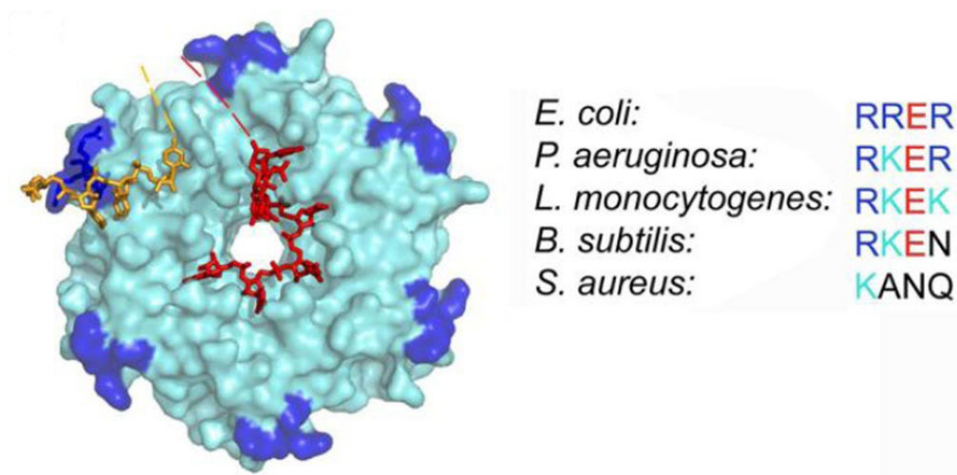


Figure 13. Basic patches on the lateral rim of the *E. coli* Hfq hexamer. *E. coli* Hfq (surface model, in cyan and blue) in complex with RydC sRNA (stick model, in red and yellow). The basic arginine patches (blue surface) are interacting with the 5′CUUC motif (in yellow) of the RydC sRNA, while the U-rich motif (in red) of the sRNA is associated with the Hfq proximal side. The rest of the sRNA is omitted for clarity (left panel). The sequence of the basic arginine patches of Hfq proteins from different bacteria is shown at the right. Figure taken from Zheng *et al.* (2016).

The third RNA binding surface consists of a basic arginine rich-patch on the lateral rim of the Hfq-hexamer, which was found to interact with the internal U/A-rich regions of both sRNAs and mRNAs (Sauer *et al.*, 2012; Panja *et al.*, 2013; Peng *et al.*, 2014; Schu *et al.*, 2015; Santiago-Frangos and Woodson, 2018; Figure 13). In addition to promoting base-pairing (Panja *et al.*, 2013, 2015; Zheng *et al.*, 2016), the lateral rim surface is deemed to aid in positioning of the interacting RNAs (Peng *et al.*, 2014). A recent study uncovered the importance of the number of arginines present in the lateral rim patch for RNA annealing (Zheng *et al.*, 2016). Hfq from *E. coli* and from *P. aeruginosa* containing arginine-rich rim patches (RRER or RKER) demonstrated strong RNA annealing activity. This is in contrast to Hfq from *B. subtilis* and *S. aureus* that carry arginine-poor rim patches (RKEN or KANQ) and show no detectable RNA annealing activity (Zheng *et al.*, 2016).

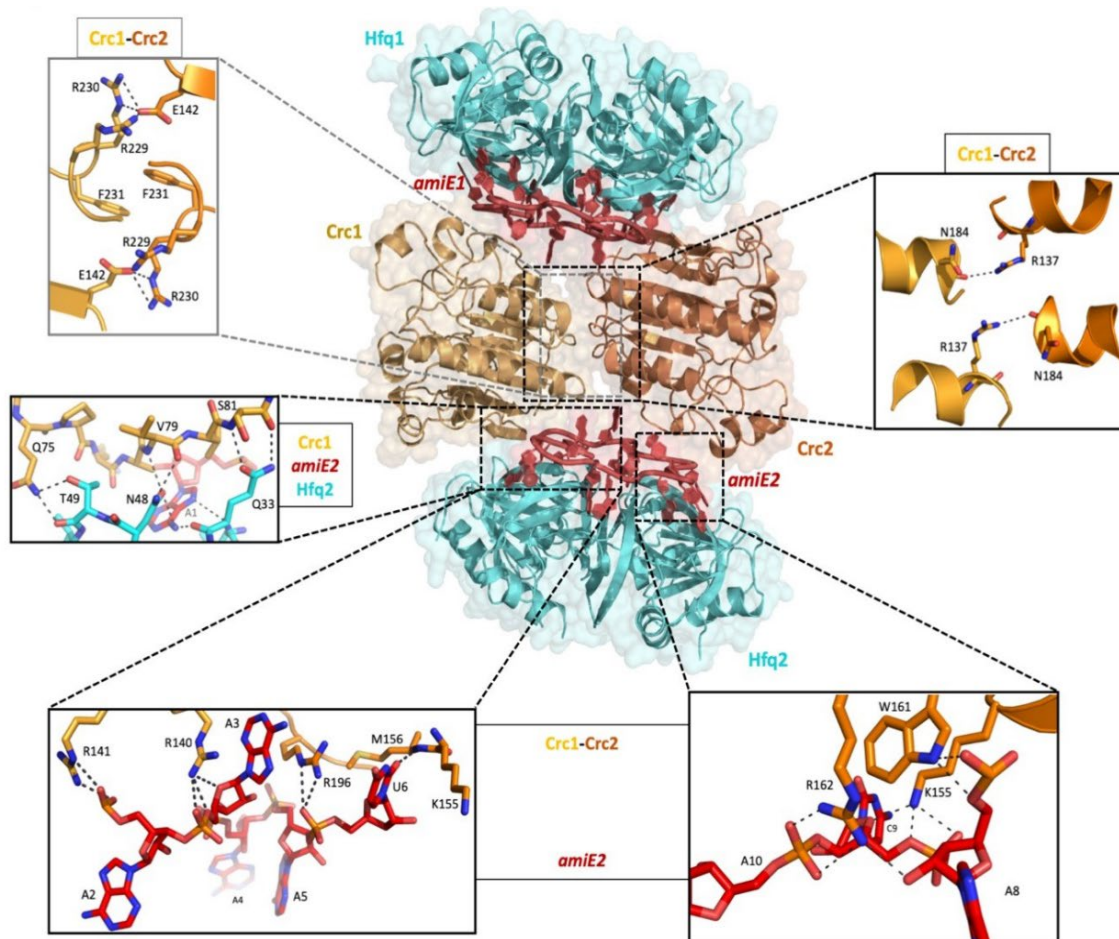


Figure 14. Structure of the Hfq/Crc repressive complex formed at the 5'-proximal Hfq binding site of *amiE* mRNA.

Quaternary structure of the repressive ribonucleoprotein complex formed with two *amiE* mRNA octadecamers, *amiE1* and

amiE2 (in red). Either *amiE* stretch, comprising the ribosome binding site, is sandwiched between two Hfq hexamers, Hfq1 and Hfq2 (in cyan) and two molecules of Crc, Crc1 and Crc2 (in yellow and ochre brown). Key bonds and interactions, which are important for the Crc interactions (top insets), Hfq-Crc interactions (middle left inset) and Crc-*amiE* mRNA interactions (bottom insets) are highlighted in the respective insets. Figure taken from Pei *et al.* (2019).

The intrinsically disordered and flexible C-terminus of Hfq varies significantly in sequence and length between different bacterial species (Beich-Frandsen *et al.*, 2011; Sauer, 2013). Despite some initial controversial studies, it is suggested to mediate the interaction with several sRNAs (Salim *et al.*, 2012; Vincent *et al.*, 2012; Dimastrogiovanni *et al.*, 2014), as well as with *hfq* mRNA (Vecerek *et al.*, 2008; Robinson *et al.*, 2014). The acidic residues of the C-terminus were shown to interact with the basic arginine residues on the lateral rim of the Hfq-hexamer, acting as a competitor for interacting RNAs, which in turn appears to drive the fast displacement of sRNA-mRNA-duplexes and (re)cycling of Hfq, and aids to substrate specificity in various bacterial species (Santiago-Frangos *et al.*, 2016, 2019; Woodson *et al.*, 2018). In addition, the C-terminus seems to contribute to the stability of the Hfq-hexamer (Arлуison *et al.*, 2004), as well as to the localization of Hfq within membrane-associated coiled structures *in vivo* (Fortas *et al.*, 2015; Malabirade *et al.*, 2017b).

Aside from its well-established role in sRNA-mediated riboregulation, Hfq was proposed to have further roles (Kavita *et al.*, 2018; dos Santos *et al.*, 2019; Quendera *et al.*, 2020). For instance, Hfq can bind to A-rich sequences at or in the close proximity of the rbs of some target mRNAs, and act as a direct translational repressor (Sonnleitner and Bläsi, 2014; Figure 14). In addition, Hfq can also bind to rRNAs and is supposed to be involved in rRNA processing, ribosome biogenesis and maturation and thus, its presence is critical for translational fidelity in *E. coli* (Andrade *et al.*, 2018; Sharma *et al.*, 2018). Moreover, Hfq was found to bind D-stem and the T-stem of the tRNAs with its proximal surface at a site distinct from its sRNA binding site (Lee and Feig, 2008). It was previously shown that Hfq can not only interact with RNA, but also with DNA (Cech *et al.*, 2016). The C-terminus of the *E. coli* Hfq can bind to a DNA duplex and is able to promote the compaction of DNA segments (Jiang *et al.*, 2015;

Malabirade *et al.*, 2017a). However, the effect on the overall DNA topology seems to be rather indirect and is probably governed *via* Hfq-sRNA-mediated regulation of other nucleoid-associated protein(s) (Malabirade *et al.*, 2018).

Hfq also directly or indirectly associates with different proteins and forms ribonucleoprotein complexes implicated in the modulation of the expression of specific subset of genes (Morita *et al.*, 2005; Van den Bossche *et al.*, 2014; Sonnleitner *et al.*, 2018; Caillet *et al.*, 2019; Pei *et al.*, 2019). For example, in *E. coli* it was found to associate with members of RNA degradation machinery, *e.g.* RNase E (Morita *et al.*, 2005; Ikeda *et al.*, 2011), polyA-polymerase (Le Derout *et al.*, 2003; Mohanty *et al.*, 2004), exonuclease polynucleotide phosphorylase PNPase (Bandyra *et al.*, 2016), as well as with termination factor Rho (Rabhi *et al.*, 2011) and elongation factor EF-Tu (Caillet *et al.*, 2019). In *P. aeruginosa*, Hfq associates with the components of RNA degradosome (Van den Bossche *et al.*, 2014) and with the catabolite repression control protein Crc (Sonnleitner *et al.*, 2018; Pei *et al.*, 2019; Figure 14).

In *P. aeruginosa*, Hfq serves as a pleiotropic post-transcriptional regulator of virulence and metabolism (Sonnleitner *et al.*, 2003, 2006, 2017; Sonnleitner and Bläsi, 2014; Ferrara *et al.*, 2015; Lu *et al.*, 2016; Zhang *et al.*, 2017), which often acts in concert with Crc (Kambara *et al.*, 2018; Sonnleitner *et al.*, 2018; Pei *et al.*, 2019; Figure 15). Crc functions as a co-repressor which binds to Hfq/target mRNA and promotes the stabilization of the assembly, without significantly increasing the affinity of Hfq for the interacting mRNA (Sonnleitner *et al.*, 2018). Recent structural studies performed with a short octadecamer derived from the 5'UTR of the *amiE* gene revealed that the two RNA segments are sandwiched between Hfq and Crc (Pei *et al.*, 2019; Figure 14), which readily explained the increase in translational repression in the presence of Crc, when compared to Hfq alone (Sonnleitner and Bläsi, 2014). Moreover, Hfq can bind to the regulatory RNA CrcZ, which counteracts Hfq/Crc-mediated translational repression of genes involved in carbon metabolism (Sonnleitner and Bläsi, 2014), as well as Hfq/sRNA-mediated riboregulation (Sonnleitner *et al.*, 2017; Figure 21-22).

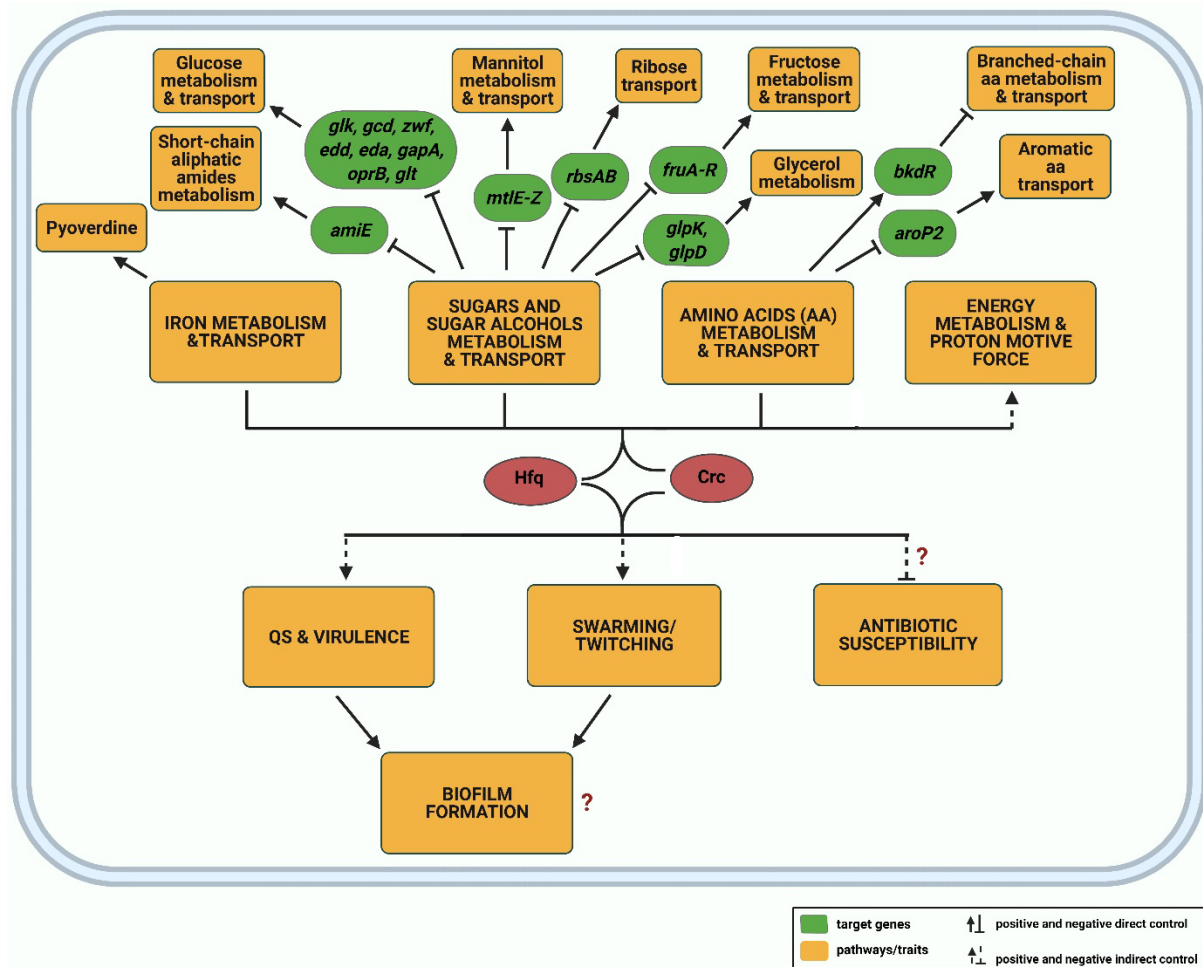


Figure 15. Pleiotropic effects of Hfq and Crc on metabolism and virulence in *P. aeruginosa*. Hfq and Crc proteins (in red) are both necessary for tight translational repression of genes involved in carbon, iron and energy metabolism in *P. aeruginosa* (upper panel). In addition, they both appear to be implicated in direct (straight lines) or indirect (dashed lines) regulation of QS, virulence, antibiotic susceptibility and biofilm formation (lower panel). The Hfq/Crc-mediated regulation is antagonized by the Hfq- or the Hfq/Crc-binding RNA CrcZ, which is further explained in Figures 21 and 22 and in the text below. Arrow ↓: positive control; ⊥: negative control. The red question marks indicate the pathways/traits that were unknown to be regulated by Hfq/Crc before this work was started. The scheme was drawn according to Sonnleitner *et al.* (2006, 2012b, 2018), Linares *et al.* (2010), Kambara *et al.* (2018) and Corona *et al.* (2018).

2.6.3. Regulatory RNAs

High-throughput RNA sequencing approaches revolutionized studies on bacterial transcriptomes and identified numerous regulatory RNAs, many of which are fundamental for the regulation of

metabolism, virulence and the adaptation to varying environmental cues (Toledo-Arana *et al.*, 2007; Waters and Storz, 2009; Sorek and Cossart, 2010; Wagner and Romby, 2015; Dar *et al.*, 2016; Holmqvist and Wagner, 2017; Hör and Vogel, 2017; Durica-Mitic *et al.*, 2018; Chakravarty and Massé, 2019; Diallo and Provost, 2020; González Plaza, 2020; Iosub *et al.*, 2020; Mediati *et al.*, 2021).

Bacterial regulatory RNAs, are mostly non-coding and usually synthesized under certain physiological conditions induced by stress (Holmqvist and Wagner, 2017). Regulatory RNAs can rewire gene expression by various modes of actions. Once the trigger is lost, the regulatory RNA is often co-degraded with the target mRNA (Massé *et al.*, 2003). Regulatory RNAs are diverse and can act at all levels of gene expression, by (i) controlling DNA maintenance and silencing (Itoh and Tomizawa, 1980; Kittle *et al.*, 1989; Marraffini and Sontheimer, 2008), (ii) activating or repressing transcription (Wassarman, 2007a, 2007b, 2018; Sedlyarova *et al.*, 2016; Chen *et al.*, 2019), (iii) affecting translation and/or stability of target mRNAs (Waters and Storz, 2009; Storz *et al.*, 2011; Papenfort and Vanderpool, 2015; Wagner and Romby, 2015; Jørgensen *et al.*, 2020), (iv) modulating the function of proteins (Babitzke and Romeo, 2007; Wassarman, 2007a, 2007b, 2018; Lapouge *et al.*, 2008; Sonnleitner and Bläsi, 2014; Romeo and Babitzke, 2018), or (v) by sequestering other sRNAs (Azam and Vanderpool, 2015; Miyakoshi *et al.*, 2015; Denham, 2020). Depending on their mode of action, regulatory RNAs can be classified into seven major subgroups: small base-pairing RNAs (sRNAs), dual-function (bifunctional) RNAs, protein-binding RNAs, RNA sponges, riboswitches, RNA thermometers and CRISPR RNAs.

2.6.3.1. Base-pairing RNAs

Base-pairing sRNAs can be divided into two categories: *cis*-encoded and *trans*-encoded. The *cis*-encoded RNAs or *anti-sense* RNAs (asRNAs) are transcribed from the opposite strand of a gene they are regulating and thus, are fully complementary to the target mRNA (Brantl, 2007; Thomason and Storz, 2010; Georg and Hess, 2011). Commonly employed mechanisms of regulation by asRNAs entail

(i) the inhibition of translation by interfering with ribosome binding (Figure 16A), (ii) transcriptional attenuation (Figure 16E) and (iii) altering the target RNA stability by promoting its processing and/or degradation (Figure 16F-H) (Wagner *et al.*, 2002; Brantl, 2007; Georg and Hess, 2011; Toledo-Arana and Lasa, 2020). In addition, the transcription of the asRNA itself can interfere with the transcription of the target by causing occlusion of a “weaker” promoter of the target by an RNA polymerase complex originating from a “stronger” promoter of the asRNA. Alternatively, the transcription of the asRNA can cause the collision and/or displacement of one of the RNA polymerases from two intersecting elongation complexes, also known as “sitting-duck” interference (Shearwin *et al.*, 2005; Georg and Hess, 2011). Furthermore, another concept of anti-sense regulation, termed as excludon, was recently coined (Sesto *et al.*, 2013). The excludon represents a long RNA, which acts as both, a coding mRNA and an asRNA to repress the expression of the overlapping transcript from a contiguous gene (Sesto *et al.*, 2013; Toledo-Arana and Lasa, 2020).

Although transcriptome analyses show a high pervasiveness of asRNAs transcription, the biological function of only a few asRNA was elucidated until now (Lybecker *et al.*, 2014; Georg and Hess, 2018). Most bacterial asRNAs are transcribed from mobile genetic elements (*e.g.* transposons, plasmids) and are involved in their maintenance and silencing (Brantl, 2002, 2007; Wagner *et al.*, 2002). The first characterized *cis*-encoded RNA in bacteria was shown to regulate the *E. coli* ColE1 replication (Itoh and Tomizawa, 1980). Later, a *cis*-encoded RNA-OUT was revealed to control the *E. coli* Tn10/IS10 transposase (Kittle *et al.*, 1989). Nevertheless, asRNAs can also be chromosomally encoded and often serve as anti-toxins that alter the expression of their cognate toxin gene as a part of type I toxin-antitoxin systems (Gerdes and Wagner, 2007; Wen and Fozo, 2014).

Trans-encoded sRNAs are commonly encoded at a locus that is apart from the target gene they regulate. Therefore, they often exhibit limited sequence complementarity with their target mRNA (Waters and Storz, 2009; Adams and Storz, 2020; Jørgensen *et al.*, 2020). *Trans*-encoded sRNAs

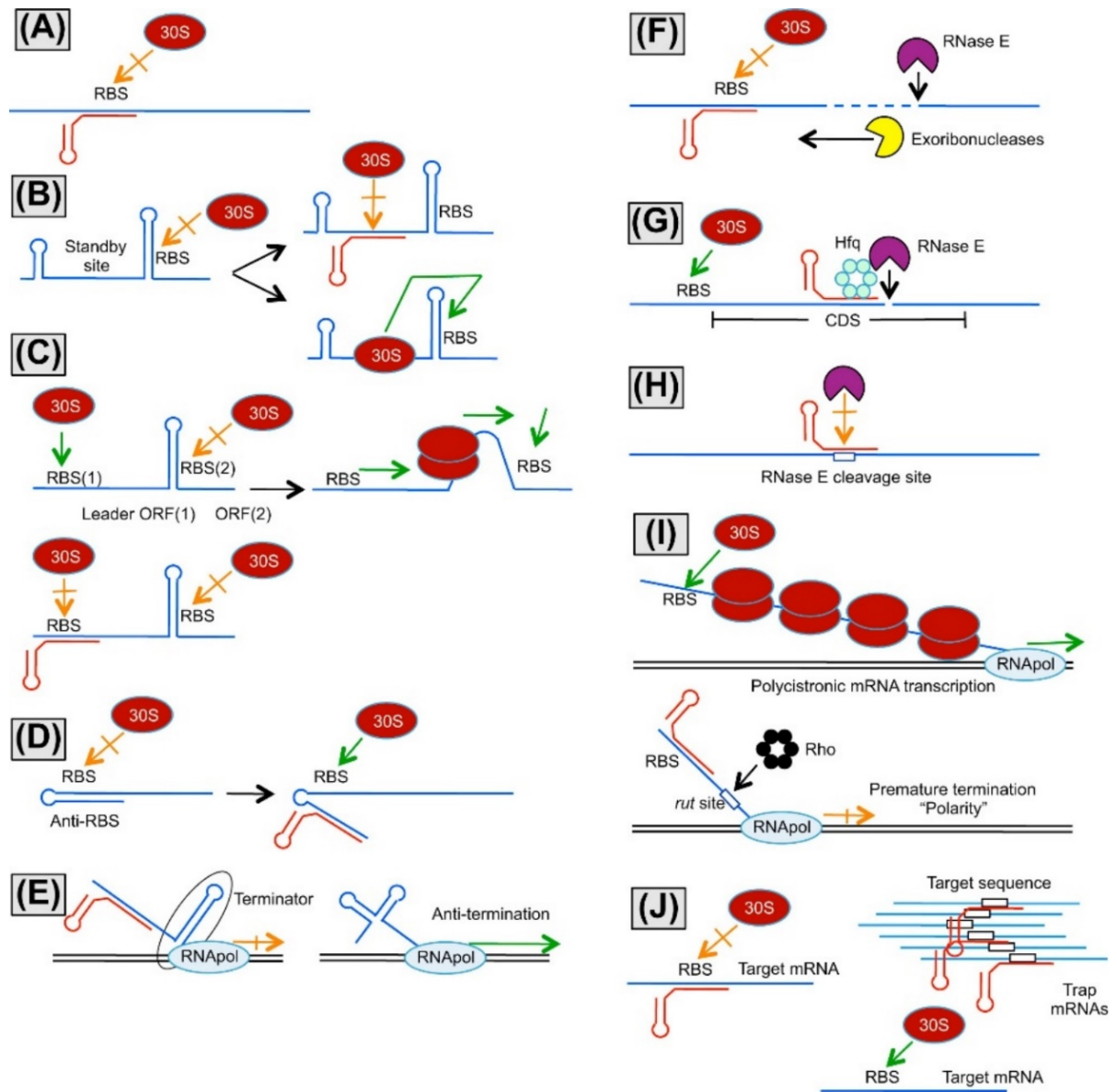


Figure 16. Different mechanisms of regulation by base-pairing RNAs. (A) Base-pairing of a sRNA in the close vicinity of the ribosomal binding site (RBS) of the target mRNA prevents binding of the 30S ribosomal subunit (30S), and thereby hampers translation initiation (e.g. *MicA*) (Udekwi *et al.*, 2005; Morita *et al.*, 2006). (B) The secondary structure prevents binding of the 30S to the RBS and the 30S subunit may bind to a standby site. The transient breathing enables the access to the RBS and translation initiation (lower panel). However, if the sRNA base-pairs with the standby site, this in turn can prevent the binding of 30S and subsequent translation (upper panel) (e.g. *IstR-1*) (Darfeuille *et al.*, 2007). (C) Translational coupling to an upstream leader open reading frame (ORF) sequence (ORF (1)) permits the translation of both ORF1 and the downstream ORF2, whose RBS (RBS (2)) is not accessible due to an inhibitory secondary structure (Sonnleitner *et al.*, 2011; upper panel). In opposite, base-pairing of an sRNA to the RBS (RBS(1)) of the ORF1 impedes the translation of both ORFs (lower panel) (e.g. *RyhB*) (Vecerek *et al.*, 2007). (D) The activation of translation through anti-antisense mechanism: the RBS is occluded by an

inhibitory secondary structure (anti-RBS), which hampers 30S binding. Base-pairing of an sRNA to the anti-RBS resolves the inhibitory secondary structure and promotes translation (*e.g.* RnaIII, DsrA) (Morfeldt *et al.*, 1995; Majdalani *et al.*, 1998; Papenfort and Vanderpool, 2015). **(E)** The binding of the sRNA can drive transcriptional attenuation by promoting premature transcriptional termination. In the absence of an sRNA, the RNA polymerase (RNAPol, blue oval) ignores the termination structure and continues the elongation of the transcript (anti-termination; right). However, upon base-pairing of the sRNA, the termination structure is further stabilized, leading to premature termination (left; Giangrossi *et al.*, 2010). **(F)** Translational inhibition by the sRNA is often accompanied by target mRNA cleavage and decay by RNases (*e.g.* SgrS) (Massé *et al.*, 2003; Moll *et al.*, 2003a; Vanderpool and Gottesman, 2004; Kaberdin and Bläsi, 2006). **(G)** A “decay only” mechanism: an sRNA, associated with the RNA chaperone Hfq, base-pairs with the coding sequence (CDS) of the target mRNA and promotes its RNase E-dependent degradation, without impeding translation initiation (*e.g.* MicC) (Pfeiffer *et al.*, 2009; Bandyra *et al.*, 2012). **(H)** An sRNA base-pairs to an RNase E cleavage site, occluding it and thus hinders its degradation (*e.g.* RydC) (Fröhlich *et al.*, 2013). **(I)** An sRNA induces transcriptional polarity and operon-wide effects by co-transcriptionally base-pairing with the RBS of an upstream ORF in the polycistronic mRNA resulting in the ribosome “stripping” of the whole operon. This in turn can disclose occluded downstream Rho utilization (rut) sites and enable binding of the termination factor Rho (black hexamer), which leads to premature termination of the whole polycistronic operon (lower panel) (*e.g.* ChiX) (Bossi *et al.*, 2012). **(J)** Small RNAs can serve as traps or sponges (anti-sRNAs) for other base-pairing sRNAs, thus antagonizing their function in target mRNA regulation (*e.g.* *chb* RNA) (Figuroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009). Base-pairing RNAs are highlighted in red and their target mRNAs are in blue. Positive regulation is indicated by green arrows and negative regulation by crossed orange arrows. Figure taken from Wagner and Romby (2015).

base-pair to their target through short and highly conserved regions, termed the “seed sequence” (Gottesman and Storz, 2011). The majority of *trans*-encoded sRNAs bind and regulate several target mRNAs, some sRNAs being able to fine-tune the expression of more than 30 genes (Prévost *et al.*, 2007). In addition, many *trans*-encoded sRNAs in Gram-negative bacteria require RNA chaperones, such as Hfq or ProQ for their function (Vogel and Luisi, 2011; Van Assche *et al.*, 2015; Olejniczak and Storz, 2017; Holmqvist and Vogel, 2018). However, RNA chaperones, especially Hfq, seem to be largely dispensable in sRNA-mediated regulation in Gram-positive bacteria (Bohn *et al.*, 2007; Jousset *et al.*, 2009; Nielsen *et al.*, 2010; Rochat *et al.*, 2015).

Base-pairing of *trans*-encoded sRNAs to the target mRNA commonly results in either direct or indirect translational inhibition and/or mRNA degradation (De Lay *et al.*, 2013), or direct or indirect translational activation and/or the prevention of target degradation (Soper *et al.*, 2010). In case of negative translational regulation, the sRNA usually base-pairs in the close vicinity of the rbs of the target mRNA. This base-pairing normally interferes with ribosome loading on the target mRNA followed by translational inhibition (Morita *et al.*, 2006; Figure 16A), and subsequent mRNA decay by RNases (Massé *et al.*, 2003; Moll *et al.*, 2003a; Kaberdin and Bläsi, 2006; Figure 16F). In contrast, *trans*-encoded sRNAs can also promote translational activation, typically *via* an anti-antisense mechanism by base-pairing to inhibitory stem-loop structures in the 5'UTR. This in turn leads to structural remodelling which can free the rbs for translation initiation (Morfeldt *et al.*, 1995; Majdalani *et al.*, 1998; Papenfort and Vanderpool, 2015; Figure 16D). In addition, *trans*-encoded sRNA can indirectly promote or impede the translation of target mRNAs. Indirect regulation often occurs through translational coupling, whereby the translation of the target mRNA is positively or negatively coupled to an *upstream open reading frame (uof)* (Vecerek *et al.*, 2007; Sonnleitner *et al.*, 2011; Papenfort and Vanderpool, 2015). Hence, regulation of the *uof* by a sRNA can stimulate (Sonnleitner *et al.*, 2011; Figures 16C and 19) or inhibit translation of the downstream gene (Vecerek *et al.*, 2007; Figure 16C). Moreover, sRNAs do not only bind in the 5'UTRs, but also within the coding region of the mRNA target (Bouvier *et al.*, 2008; Melamed *et al.*, 2016). Base-pairing with the coding region can cause structural rearrangements, which lead to the obstruction of the rbs and impede translation initiation (Heidrich *et al.*, 2007). Alternatively, it could slow or inhibit translational elongation. In addition, base-pairing within the coding sequence can also affect the stability of the target mRNA and either promote (Pfeiffer *et al.*, 2009; Bandyra *et al.*, 2012; Figure 16G) or impede its degradation (Fröhlich *et al.*, 2013; Figure 16H).

2.6.3.2. Dual-function RNAs

Dual-function or bifunctional RNAs are a class of bacterial regulatory RNAs that function as both, a regulatory base-pairing sRNA, and an mRNA encoding a small peptide (Vanderpool *et al.*, 2011; Gimpel and Brantl, 2017; Raina *et al.*, 2018). The base-pairing sRNA and the regulatory peptide often act independently within the same pathway, however, in some examples of dual-function RNAs, they are implicated in the regulation of distinct pathways (Raina *et al.*, 2018). To date, several dual-function RNAs were characterized:

SgrS RNA is involved in regulation of glucose-phosphate stress in *Enterobacteriaceae* (Vanderpool and Gottesman, 2004; Wadler and Vanderpool, 2007).

RNAIII and Psm-mec serve as modulators of virulence and antibiotic resistance in *S. aureus* (Janzon *et al.*, 1989; Novick *et al.*, 1993; Balaban and Novick, 1995; Benito *et al.*, 2000; Verdon *et al.*, 2009; Queck *et al.*, 2009; Kaito *et al.*, 2011, 2013; Fechter *et al.*, 2014; Ikuo *et al.*, 2014; Qin *et al.*, 2016).

Pel RNA functions as a regulator of virulence genes in *Streptococcus pyogenes* (Betschel *et al.*, 1998; Mangold *et al.*, 2004).

PhrS RNA activates quorum sensing and has been implicated in regulation of the CRISPR-Cas system in *P. aeruginosa* (Sonnleitner *et al.*, 2011; Lin *et al.*, 2019).

SR1 is a dual-function sRNA from *B. subtilis* (Gimpel and Brantl, 2017). It is transcribed from a SigA-dependent promoter under gluconeogenic conditions, whereas it is repressed under glycolytic conditions mainly by CcpN and to a minor extent by CcpA (Licht *et al.*, 2005, 2008; Licht and Brantl, 2006, 2009). Via 7 complementary regions, SR1 base-pairs with *ahrC* mRNA encoding the transcriptional activator of the arginine catabolic operons *rocABC* and *rocDEF* to inhibit its translation (Heidrich *et al.*, 2006). Translational inhibition occurs by inducing structural changes around the *ahrC* rbs (Heidrich *et al.*, 2007). In addition, SR1 encodes the small peptide SR1P (39 aa) that interacts with

glyceraldehyde-3-phosphate dehydrogenase GapA (Gimpel *et al.*, 2010). This interaction increases binding of GapA to RNase J1 and significantly enhances RNase J1 activity (Gimpel and Brantl, 2016; Gimpel *et al.*, 2017).

2.6.3.3. Protein-binding RNAs

Protein-binding RNAs commonly use sequence or structural mimicry to sequester regulatory proteins and modulate their activity. Only a few examples of bacterial protein-binding RNAs are known. One such example is 6S RNA which is present in a broad range of bacteria (Barrick *et al.*, 2005), and was studied extensively in *E. coli* and *B. subtilis* (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2005; Gildehaus *et al.*, 2007; Cavanagh *et al.*, 2008; Klocko and Wassarman, 2009; Cavanagh and Wassarman, 2014; Steuten *et al.*, 2014; Chen *et al.*, 2017; Wassarman, 2018; Figure 17A). 6S RNA mimics the open promoter complex and directly binds to region 4 of the *E. coli* DNA-dependent RNA polymerase sigma 70 (σ^{70}) holoenzyme titrating it from its target promoters (Wassarman and Storz, 2000; Gildehaus *et al.*, 2007; Cavanagh *et al.*, 2008; Klocko and Wassarman, 2009; Chen *et al.*, 2017). The RNA polymerase holoenzyme uses bound 6S RNA as a template for RNA synthesis and thus acts as an RNA-dependent RNA polymerase (Wassarman and Saecker, 2006; Gildehaus *et al.*, 2007). The resulting short transcripts (pRNAs) vary in size (Wassarman and Saecker, 2006). When pRNAs surpass the ≈ 13 nt length limit, they bind to 6S RNA causing structural rearrangements, which in turn leads to dissociation of the 6S RNA:RNA polymerase complex (Wassarman and Saecker, 2006; Gildehaus *et al.*, 2007). In *E. coli*, the 6S RNA steady-state levels increase towards stationary phase when the transcription of genes controlled by alternative sigma factors enable an adaptation to occurring nutrient and NTP limitations (Trotochaud and Wassarman, 2005). Hence, by titrating the *E. coli* σ^{70} -holoenzyme, 6S RNA contributes to the transcriptional shift during the transition from the exponential to the stationary phase (Trotochaud and Wassarman, 2005; Wassarman, 2007a, 2007b; Figure 17A). As nutrients become available and NTP levels increase, 6S RNA-directed pRNA synthesis takes place,

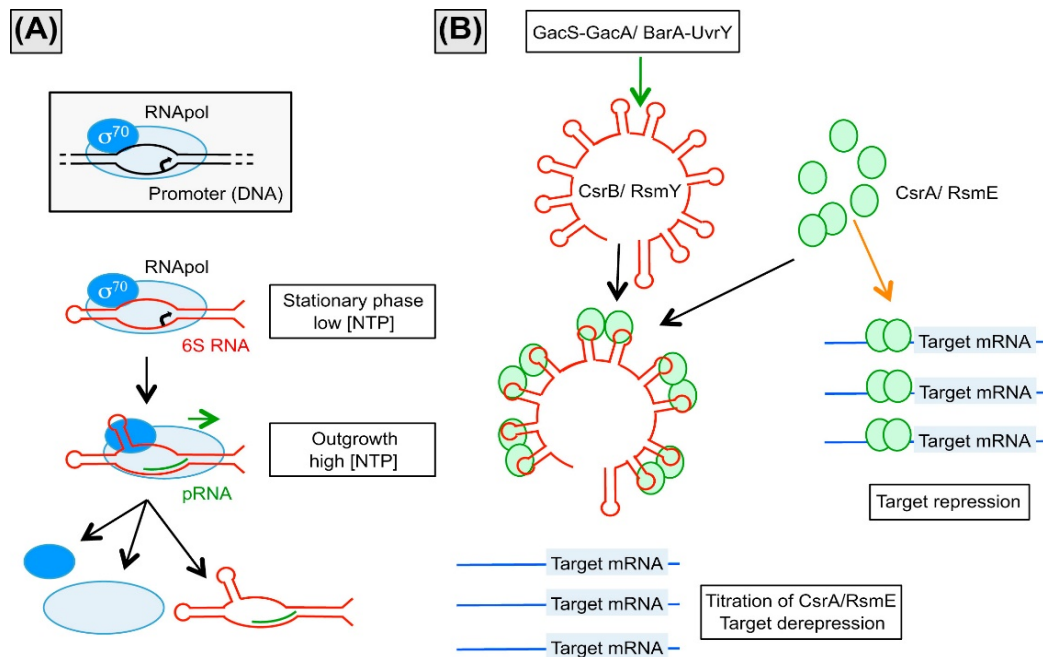


Figure 17. Regulatory mechanisms of protein-binding RNAs. Protein-binding RNAs usually sequester regulatory proteins and modulate their activity by structurally imitating proteins' target DNA, mRNAs or other sRNAs. **(A)** During the transition to stationary phase in *E. coli*, when the concentration of nucleotide triphosphates (NTPs) decreases, 6S RNA (in red) mimics the open promoter complex and directly binds to the DNA-dependent RNA polymerase sigma 70 (σ^{70}) holoenzyme (light and dark blue circle, respectively) titrating it from its target promoters (Trottochaud and Wassarman, 2005; Wassarman, 2007a, 2007b). Upon replenishment of NTPs during outgrowth, the pRNA (in green) binds to 6S RNA and induces structural rearrangements, which result in dissociation of the 6S RNA:RNA polymerase complex (Trottochaud and Wassarman, 2005; Wassarman, 2007a, 2007b). **(B)** CsrB/C and RsmV/W/Y/Z RNA families (in red) are activated by two-component systems (black square). They contain multiple binding sites for the RNA binding protein CsrA in *E. coli* and its homologues in *P. aeruginosa* and *P. fluorescens*, RsmA, RsmN/F and RsmE (green circle), respectively. In turn, this leads to the titration of CsrA and Rsm proteins and subsequently to de-repression of CsrA/Rsm controlled mRNAs (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008; Romeo and Babitzke, 2018). Figure taken from Wagner and Romby (2015).

resulting in the dissociation of the σ^{70} -holoenzyme from 6S RNA and outgrowth from stationary phase (Trottochaud and Wassarman, 2005; Wassarman, 2007a, 2007b; Figure 17A). The increase in 6S RNA expression during stationary phase is not observed in all bacteria (Barrick *et al.*, 2005). For instance, in *Helicobacter pylori*, the 6S RNA steady-state levels increase during early to mid-exponential phase

and remain rather stable up to late stationary phase (Sharma *et al.*, 2010). Moreover, some bacteria, like *Synechococcus* spp., show even diminished 6S RNA levels in stationary phase (Watanabe *et al.*, 1997; Barrick *et al.*, 2005). This demonstrates the versatile and complex nature of 6S RNA-mediated fine-tuning of transcription, which appears not to be strictly linked with the stationary phase of bacterial cell growth.

The sRNA GlmY sRNA in *Enterobacteriaceae* also acts by protein binding (Urban *et al.*, 2007). GlmY sRNA mimics the base-pairing sRNA GlmZ and acts as a decoy for the RNase E adaptor protein RapZ and thereby impedes the RapZ-mediated recruitment of RNase E, and the degradation of GlmZ (Göpel *et al.*, 2013, 2014). In turn, GlmZ sRNA base-pairs with and activates in an Hfq-dependent manner *glmS* mRNA, encoding an enzyme mediating glucosamine-6-phosphate homeostasis and cell wall synthesis (Kalamorz *et al.*, 2007; Urban and Vogel, 2008; Göpel *et al.*, 2014, 2015).

Further examples of protein-binding RNAs are the CsrB/C and RsmV/W/Y/Z RNA families, which serve as a molecular sink of CsrA in *E. coli* and its homologues in *P. aeruginosa*, RsmA and RsmN/F, respectively (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008; Romeo and Babitzke, 2018; Figure 17B). The CrcY/X/Z RNA family represents an additional family of protein-binding RNAs that act in *Pseudomonads* as important regulators of the RNA chaperone Hfq (Sonnleitner *et al.*, 2009, 2017, 2018; Moreno *et al.*, 2012, 2015; Filiatrault *et al.*, 2013; Sonnleitner and Bläsi, 2014; La Rosa *et al.*, 2015).

2.6.3.4. RNA sponges

RNA sponges, anti-sRNAs, RNA traps or competing endogenous RNAs (ceRNAs) interact with other sRNAs and function as decoys. Thereby, they antagonize their function by preventing them from binding to their mRNA targets, and often by fostering their decay (Vogel, 2009; Azam and Vanderpool, 2015; Miyakoshi *et al.*, 2015; Bossi and Figueroa-Bossi, 2016; Figueroa-Bossi and Bossi, 2018; Kavita

et al., 2018; Gröll and Massé, 2019; Denham, 2020; Melamed *et al.*, 2020). Some RNA sponges seem to be constitutively expressed, while others are induced upon environmental signals (Gröll and Massé, 2019). RNA sponges usually originate either from processed mRNAs, UTRs or externally transcribed spacers, intergenic regions, internal promoters, anti-sense RNA and/or as independent transcripts, which are synthesized under control of their own promoter and terminator (Denham, 2020). In addition, there seems to be at least one RNA sponge which is part of a subgroup of regulatory RNAs recently denoted as decay-generated non-coding RNAs (decRNAs) (Dar and Sorek, 2018). The decRNAs are excised from within the protein coding regions of mRNA by *E. coli* RNase E as a result of a non-uniform mRNA decay (Dar and Sorek, 2018).

One well-studied example of an RNA sponge in *E. coli* and *Salmonella enterica* represents the intergenic *chb* RNA, whose expression is induced in the presence of chitooligosaccharides (Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009). The *chb* RNA acts as an anti-RNA of the constitutively expressed ChiX (formerly SroB, RybC or MicM) sRNA which binds to and represses *chiP* (formerly *ybfM*) mRNA, encoding a porin involved in the uptake of chitooligosaccharides (Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009; Rasmussen *et al.*, 2009). The *chb* RNA most probably destabilizes ChiX sRNA by invading the stem of its transcription terminator hairpin and is concomitantly degraded together with ChiX (Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009; Figure 16J). Other well-characterized representatives of RNA sponges are the SroC RNA, mRNA-derived multi-target sponge of GcvB and MgrR sRNAs (Miyakoshi *et al.*, 2015; Acuña *et al.*, 2016) and a 3'externally transcribed spacer of tRNA-Leu, 3'ETS_{leuZ} which acts as a decoy for RyhB and RybB sRNAs to reduce transcriptional noise (Lalaouna *et al.*, 2015).

2.6.3.5. Riboswitches

Riboswitches are *cis*-encoded, *cis*-acting 5'UTR RNA structures that undergo conformational

alterations upon changes in pH or ligand-binding in response to environmental cues (Nou and Kadner, 2000; Nahvi *et al.*, 2002; Winkler *et al.*, 2002; Mandal and Breaker, 2004; Henkin, 2008; Nechooshtan *et al.*, 2009; Smith *et al.*, 2010; Bastet *et al.*, 2011; Sherwood and Henkin, 2016). They usually comprise two functional domains: a ligand-binding domain and a regulatory expression domain (Mandal and Breaker, 2004; Nudler and Mironov, 2004; Henkin, 2008; Montange and Batey, 2008; Bastet *et al.*, 2011). The activity of riboswitches is modulated by changes in the concentration of their ligands, most commonly metal ions, metabolites or small molecules such as amino acids, carbohydrates, nucleotides or cofactors (Nou and Kadner, 2000; Nahvi *et al.*, 2002; Winkler *et al.*, 2002; Barrick and Breaker, 2007; Regulski *et al.*, 2008; Spinelli *et al.*, 2008; Sudarsan *et al.*, 2008; Haller *et al.*, 2011; Peselis and Serganov, 2014; Hernandez-Valdes *et al.*, 2020). Riboswitches function as regulators at the transcriptional or translational level, modulating the processes of transcriptional termination and anti-termination, stability and translation of distinct genes, encoding functions for the metabolism or uptake of metal ions, cofactors, nucleotides or amino acids (Nou and Kadner, 2000; Winkler *et al.*, 2002; Nahvi *et al.*, 2002; Nudler and Mironov, 2004; Barrick and Breaker, 2007; Henkin, 2008; Smith *et al.*, 2010; Bastet *et al.*, 2011; Santangelo and Artsimovitch, 2011; Lemay *et al.*, 2011; Hollands *et al.*, 2012; Peselis and Serganov, 2014; Sherwood and Henkin, 2016; Hernandez-Valdes *et al.*, 2020).

2.6.3.6. RNA thermometers

RNA thermometers are RNA regulatory elements that reside in the 5'UTR of an mRNA, which act in *cis* in response to temperature alterations (Kortmann and Narberhaus, 2012; Righetti and Narberhaus, 2014; Loh *et al.*, 2018). They commonly consist of a stem-loop structure that can occlude the rbs. At lower temperatures, RNA thermometers often impede the translation of genes involved in virulence and/or heat shock response. When the temperature increases, these inhibitory structures are temporally relieved by a zipper-like mechanism and the translation of virulence (37°C) and heat shock genes ($\geq 40^\circ\text{C}$) can commence (Kortmann and Narberhaus, 2012; Righetti and Narberhaus, 2014; Loh

et al., 2018). In contrast, in the case of the cold shock RNA chaperone CspA mRNA, a switch to a “cold shock structure” occurs upon decrease of the temperature ($\leq 10^{\circ}\text{C}$). This switch increases the accessibility for ribosomes and the stability of *cspA* mRNA, which in turn leads to enhanced translation (Giuliodori *et al.*, 2010; Kortmann and Narberhaus, 2012).

2.6.3.7. CRISPR RNAs

Clustered *regularly interspaced short palindromic repeats* (CRISPR) RNAs evolved as a part of the bacterial and archaeal adaptive immune response system to combat phage infection. They have been shown to confer immunity against foreign DNAs or RNAs originating from viruses, plasmids, prophages and/or transposons (Ishino *et al.*, 1987; Pourcel *et al.*, 2005; Lillestøl *et al.*, 2006; Makarova *et al.*, 2006, 2013; Barrangou *et al.*, 2007; Sorek *et al.*, 2008; Horvath and Barrangou, 2010; Jinek *et al.*, 2012; Westra *et al.*, 2016; Koonin and Makarova, 2019). The system utilizes short non-coding single-guide RNA (sgRNA), which recognizes and binds to the complementary target nucleic sequence and acts as a guide for the CRISPR-associated (cas) nuclease, which endonucleolytically cleaves and inactivates the target DNA or RNA (Deltcheva *et al.*, 2011; Jinek *et al.*, 2012; Makarova *et al.*, 2013; van der Oost *et al.*, 2014; Charpentier *et al.*, 2015).

2.6.4. Regulatory RNAs in *P. aeruginosa*

Several different approaches were employed for the identification of *P. aeruginosa* sRNAs including *in silico* predictions (Wilderman *et al.*, 2004; Livny *et al.*, 2006), co-purification with Hfq protein (Sonnleitner *et al.*, 2008) and high-throughput RNAseq analyses under different growth conditions (Dötsch *et al.*, 2012; Ferrara *et al.*, 2012; Gómez-Lozano *et al.*, 2012; Wurtzel *et al.*, 2012). A total of 573 sRNAs were detected in strain PAO1 and 233 sRNAs in the clinical strain PA14 (Ferrara *et al.*, 2012; Gomez-Lozano *et al.*, 2015; Pita *et al.*, 2018). Although 126 sRNAs were identified to be commonly

present in both strains, *in silico* predictions suggest that the actual number of shared sRNA is larger (Li *et al.*, 2013b; Gomez-Lozano *et al.*, 2015; Pita *et al.*, 2018). Only a few of these RNAs have been functionally characterized. These include regulators of virulence and biofilm formation (ErsA, Sr0161, Sr006, PrrF1/2, PrrH, ReaL, RgsA, Pail, NrsZ and RsmV/W/Y/Z), carbon catabolite repression (CrcZ), iron metabolism (PrrF1/2 and PrrH), nitrogen metabolism (Pail and NrsZ), quorum sensing (PhrS, PrrF1/2 and ReaL) and antibiotic resistance (ErsA, Sr0161 and Sr006) (see below and Figures 18-21).

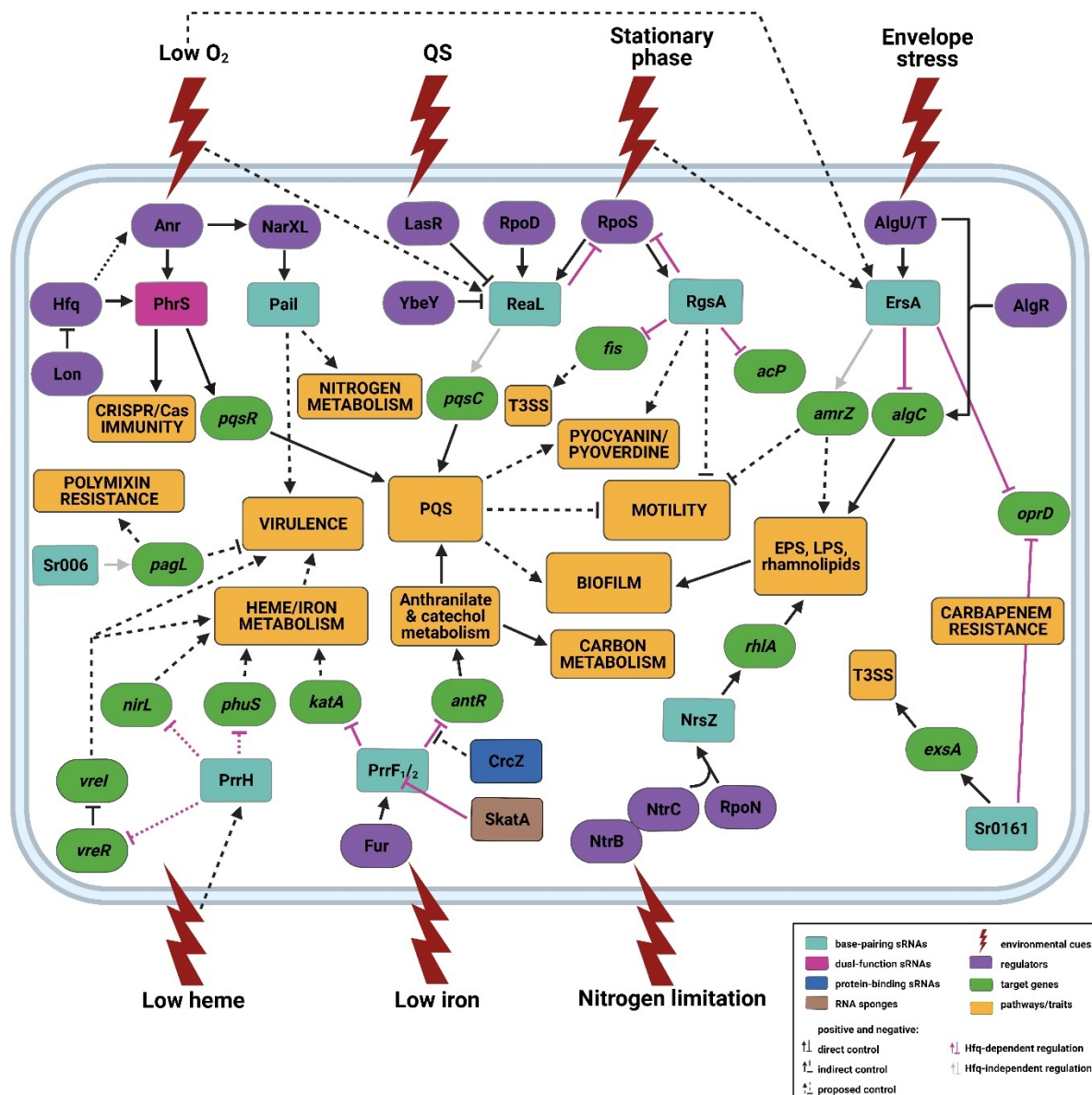


Figure 18. Regulatory network of functionally characterized *P. aeruginosa* base-pairing sRNAs. Characterized base-pairing RNAs (light malachite green boxes) and dual-function PhrS RNA (magenta box) and their respective regulators (in violet) and

targets (in green). The complex RNA-mediated regulatory network governs a number of metabolic and virulence-related processes (orange boxes) in response to different environmental cues (red flashes). See main text for details. ↓: positive control; ⊥: negative control; dashed lines: indirect control; dotted lines: proposed mechanism. Light grey lines indicate Hfq-independent regulation, whereas magenta lines highlight Hfq-dependent regulation.

2.6.4.1. Base-pairing RNAs in *P. aeruginosa*

2.6.4.1.1. ErsA

The envelope stress-responsive sRNA A (ErsA) is a *trans*-encoded sRNA which was initially identified in *P. aeruginosa* strain PAO1 as a functional orthologue of *E. coli* Spot 42 RNA (Gottesman *et al.*, 2006). ErsA was also identified in an RNAseq approach in *P. aeruginosa* strain PA14 strain as SPA0122 (Ferrara *et al.*, 2012). Transcription of *ersA* is governed by the extracytoplasmic function (ECF) sigma factor AlgU/T (σ^{22}), an envelope stress response regulator (Ferrara *et al.*, 2015). The ErsA levels are elevated upon depletion of oxygen and upon entry into stationary phase (Ferrara *et al.*, 2015; Zhang *et al.*, 2017). With the aid of Hfq, ErsA represses the translation of *algC* mRNA, encoding the bifunctional enzyme phosphomannomutase/phosphoglucomutase (Ferrara *et al.*, 2015). The AlgC enzyme is essential for the biosynthesis of alginate (Padgett and Phibbs, 1986; Sá-Correia *et al.*, 1987), exopolysaccharides Psl and Pel (Ma *et al.*, 2012), LPS (Goldberg *et al.*, 1993; Coyne *et al.*, 1994) and rhamnolipids (Olvera *et al.*, 1999), which are important constituents of the biofilm matrix during chronic infection (Deretic *et al.*, 1995; Wang *et al.*, 2014b). AlgC synthesis is activated by the alginate biosynthesis regulator AlgR and the sigma factor AlgU/T (Zielinski *et al.*, 1991, 1992; Lizewski *et al.*, 2002), which together with the sRNA ErsA are part of a feed-forward loop, which alters the AlgC enzyme levels (Ferrara *et al.*, 2015). Apart from its role in the synthesis of the biofilm matrix components through the regulation of *algC* expression, ErsA appears also to contribute to biofilm development and motility by stimulating the synthesis of the transcriptional regulator AmrZ (Falcone *et al.*, 2018). Moreover, ErsA was recently reported to be involved in pathogenesis in both acute and chronic airway infections (Ferrara *et al.*, 2020).

A recent GRILseq (Global sRNA Target Identification by Ligation and Sequencing) study revealed the *oprD* gene, encoding the outer membrane porin D, as a novel target of ErsA (Zhang *et al.*, 2017). The OprD porin constitutes an entry port for basic amino acids and peptides, as well as for carbapenem antibiotics (Trias and Nikaido, 1990a, 1990b), playing an important role in carbapenem susceptibility (Quinn *et al.*, 1986; Trias *et al.*, 1989). ErsA negatively regulates *oprD* translation, which in turn results in decreased OprD levels and reduced susceptibility to meropenem (Zhang *et al.*, 2017).

2.6.4.1.2. Sr0161

The sRNA Sr0161 was discovered in *P. aeruginosa* strain PAO1 by a GRILseq analysis (Zhang *et al.*, 2017). The *sr0161* gene is located between the genes PA0160 and *opdC*. Functional studies performed by Zhang *et al.* (2017) revealed that Sr0161, like ErsA, regulates the *oprD* gene, and thus carbapenem susceptibility. In addition, Sr0161 was suggested to regulate *opdP* mRNA, which encodes a second porin involved in carbapenem uptake (Zhang *et al.*, 2017). Another target of Sr0161 concerns the transcriptional activator of the type III secretion system, ExsA (Zhang *et al.*, 2017). Sr0161 was shown to bind to the 5'UTR of *exsA* mRNA, and to repress its translation (Zhang *et al.*, 2017). Hence, sRNA Sr0161 seems to assist in several virulence-related processes in *P. aeruginosa*.

2.6.4.1.3. Sr006

The sRNA Sr006 was originally annotated during a single-nucleotide resolution transcriptome analysis (Wurtzel *et al.*, 2012). A GRILseq approach revealed Sr006 as a positive post-transcriptional regulator of the gene encoding lipid A 3-O deacylase PagL (Zhang *et al.*, 2017). PagL deacetylates lipid A in the LPS of *P. aeruginosa* (Ernst *et al.*, 1999). The acetylated form of lipid A was previously shown to be pro-inflammatory causing severe pulmonary damage in chronically infected CF-patients (Ernst *et al.*, 1999, 2003, 2006). Sr006 interacts with the 5'UTR of *pagL* mRNA in an Hfq-independent manner

(Zhang *et al.*, 2017), which in turn increases PagL levels and elicits elevated polymyxin B resistance (Kawasaki *et al.*, 2007; Han *et al.*, 2018), and decreased pro-inflammatory signalling in *P. aeruginosa* (Moskowitz and Ernst, 2010).

2.6.4.1.4. PrrF1/2

The iron-responsive sRNAs PrrF1 and PrrF2 represent functional orthologues of the *E. coli* sRNA RyhB (Massé and Gottesman, 2002; Vecerek *et al.*, 2003; Geissmann and Touati, 2004) and *B. subtilis* FsrA sRNA (Gaballa *et al.*, 2008). PrrF1 and PrrF2 are encoded in tandem and share > 95% of sequence identity. The *prrF* genes are transcriptionally controlled by the ferric uptake regulator Fur (Wilderman *et al.*, 2004). The PrrF RNAs have been implicated in the maintenance of iron homeostasis under iron-limiting conditions by post-transcriptionally repressing genes encoding non-essential iron-containing proteins (Wilderman *et al.*, 2004; Vasil, 2007; Oglesby *et al.*, 2008; Han *et al.*, 2016; Zhang *et al.*, 2017; Arrieta-Ortiz *et al.*, 2020). PrrF-mediated riboregulation is mediated by Hfq (Sonnleitner *et al.*, 2017). A previous GRILseq study identified an RNA sponge derived from the 3' end of *kata* mRNA, encoding the major catalase in *P. aeruginosa* (Han *et al.*, 2016; Zhang *et al.*, 2017). The RNA sponge SkatA acts as a decoy for PrrF1, interfering with PrrF1 base-pairing to the 5' end of the *kata* transcript, and thus with translational repression (Han *et al.*, 2016; Zhang *et al.*, 2017). Hfq was recently shown to bind to both, the SkatA and PrrF RNAs suggesting a role for their maturation and/or regulation (Chihara *et al.*, 2019).

The PrrF RNAs are also involved in the synthesis of the *Pseudomonas* quinolone signal (PQS), a quorum sensing quinolone affecting early biofilm development and virulence (Pesci *et al.*, 1999; McKnight *et al.*, 2000; Dubern and Diggle, 2008; Hänsch *et al.*, 2014). PrrF RNAs inhibit the expression of the *antABC* and *catBCA* operons, encoding functions required for the catabolism of anthranilate, a precursor of PQS (Oglesby *et al.*, 2008). As a consequence, anthranilate is re-directed into the PQS anabolic pathway (Oglesby *et al.*, 2008). In addition, the PrrF sRNAs were shown to foster the synthesis

of other 2-alkyl-4-quinolones (AQs), which are contributing to virulence (Reinhart *et al.*, 2017). The promoting effect on AQs was recently attributed to Hfq/PrrF-mediated repression of *antR* mRNA, encoding a transcriptional activator of the *antABC* and *catBCA* operons (Sonnleitner *et al.*, 2017; Djapgne *et al.*, 2018). Thus, the indirect Prrf-dependent regulation of AQ biosynthesis represents an example of a cross-talk between iron and carbon metabolism and virulence in *P. aeruginosa*. In addition, a recent proteomic study further expanded the PrrF-dependent regulon for several iron-regulated proteins, which are involved in amino acid metabolism, twitching motility, and zinc homeostasis (Nelson *et al.*, 2019).

2.6.4.1.5. PrrH

The heme-responsive sRNA PrrH is transcribed from the tandem *prrF1/prrF2* locus through heme-dependent anti-termination of *prrF1* transcription (Oglesby-Sherrouse and Vasil, 2010). The longer PrrH RNA appears to bind to Hfq, but with lower affinity than the PrrF RNAs (Osborne *et al.*, 2014). In addition, PrrH is deemed to fine-tune the expression of genes required for heme uptake and metabolism (Oglesby-Sherrouse and Vasil, 2010). For instance, *nirL* mRNA, which was described as a putative target of PrrH RNA (Oglesby-Sherrouse and Vasil, 2010), is part of the *nirSMCFDLGHJEN* operon encoding the dissimilatory nitrite reductase NirS (cytochrome *cd*₁). The *nir* operon also contains genes of the heme *d*₁ biosynthetic pathway, which is an alternative branch of the central heme biosynthetic pathway (Kawasaki *et al.*, 1997). Thus, the predicted PrrH-mediated repression of the alternative Nir heme *d*₁ biosynthesis under heme-limiting conditions may favour the activity of the central heme biosynthetic pathway. Moreover, PrrH is predicted to regulate *phuS*, the product of which is responsible for the flux of heme through the heme-oxygenase HemO (O'Neill and Wilks, 2013; Reinhart *et al.*, 2015). Heme represents an abundant source of iron in the human host, and therefore the ability of the bacterium to uptake and utilize heme might provide a competitive advantage during infection. *In silico* analyses performed to reveal potential virulence targets for PrrH

identified *vreR* as a putative candidate (Reinhart *et al.*, 2015). VreR is an anti-sigma factor of the cell surface signalling ECF sigma factor VreI, and is thought to be involved in *P. aeruginosa* virulence (Llamas *et al.*, 2009; Quesada *et al.*, 2016; Otero-Asman *et al.*, 2020). The translational start site and coding sequence of *vreR* mRNA appear to share extensive complementarity with PrrH (Reinhart *et al.*, 2015). Moreover, the deletion of the *prfF* locus leads to a 3-fold increase in *vreR* expression, suggesting a potential role of PrrF/PrrH in its regulation (Reinhart *et al.*, 2015).

2.6.4.1.6. Real

The highly conserved sRNA Real (*regulator of alkyl quinolone*) was initially identified in *P. aeruginosa* strain PAO1 as PaeIII (Sorger-Domenigg, 2010), and as SPA0084 in strain PA14 (Ferrara *et al.*, 2012). The Real levels are elevated during stationary phase, as well as under anoxic conditions. Real transcription is governed by the “house-keeping” sigma factor RpoD (σ^{70}) and by the stationary phase sigma factor RpoS (σ^{38} , σ^S) (Carloni *et al.*, 2017). Moreover, a recent study identified Real as the first sRNA acting as a translational repressor of *rpoS* mRNA through base-pairing, thus imposing negative feedback control (Thi Bach Nguyen *et al.*, 2018). An additional layer of regulation in the Real-RpoS negative feedback loop was recently proposed. The endoribonuclease YbeY was implicated in the regulation of *rpoS* through degradation of Real (Xia *et al.*, 2020b).

Real is also involved in QS regulation, connecting the Las and PQS systems in *P. aeruginosa* (Carloni *et al.*, 2017). Las and PQS, together with the integrated quorum sensing system (IQS) and the Rhl QS system represent a hierarchically organized regulatory quorum sensing network, which mediates *P. aeruginosa* virulence during infection (Lee and Zhang, 2015). The PQS system is essential for shaping a more stress-tolerant bacterial population in response to harsh environmental conditions (Häussler and Becker, 2008; Lin *et al.*, 2018a). Moreover, PQS synthesis results in elevated biofilm formation, pyocyanin and pyoverdine synthesis (Diggle *et al.*, 2006; Yang *et al.*, 2009; Guo *et al.*, 2014),

and in decreased swarming (Guo *et al.*, 2014). These phenotypes are commonly detected during colonization of the CF lungs (Meyer *et al.*, 1996; Lau *et al.*, 2004; Guo *et al.*, 2014). In *P. aeruginosa*, PQS biosynthesis requires the functions encoded in the *pqsABCDE* and *phnAB* operons as well as the *pqsH* gene (Gallagher *et al.*, 2002). Previous reports demonstrated that LasR, the transcriptional regulator of the Las QS system, is required for PQS biosynthesis (Pesci *et al.*, 1999) by promoting *pqsH* and *pqsR* expression (Gallagher *et al.*, 2002; Schertzer *et al.*, 2009; Williams and Cámara, 2009). However, LasR also inhibits *real* transcription (Carloni *et al.*, 2017). Real activates *pqsC* translation in an Hfq-independent manner and in turn elicits elevated PQS levels and promotes establishment of PQS-dependent virulence traits (Carloni *et al.*, 2017). Therefore, LasR inhibition of *real* expression leads to diminished PQS levels and impaired PQS-related phenotypes (Carloni *et al.*, 2017). Hence, Real acts as a modulator of LasR-dependent PQS biosynthesis and virulence in *P. aeruginosa*.

2.6.4.1.7. RgsA

The sRNA RgsA was identified by two independent bioinformatic analyses in *P. aeruginosa* strain PAO1 (Livny *et al.*, 2006; Gonzalez *et al.*, 2008). Synthesis of RgsA is induced upon shift to stationary phase, and is dependent on the stationary phase sigma factor RpoS (Gonzalez *et al.*, 2008). Transcription of *rgsA* is also indirectly activated by the GacS/GacA two-component system (Gonzalez *et al.*, 2008; Lu *et al.*, 2016). In addition, RgsA requires Hfq for its stability (Sonnleitner *et al.*, 2010; Lu *et al.*, 2016). Initial results suggested that RgsA might contribute to the oxidative stress response (Gonzalez *et al.*, 2008; Park *et al.*, 2013), while another study by Lu *et al.* (2016) implicated RgsA in pyocyanin synthesis and swarming motility. RgsA was shown to inhibit translation of the *fis* and *acpP* mRNAs, encoding the global transcriptional regulator Fis and the acyl carrier protein AcpP, respectively, in a Hfq-dependent manner (Lu *et al.*, 2016). Fis is a DNA-binding and bending protein (Koch *et al.*, 1988; Schneider *et al.*, 2001), which is essential for the expression of T3SS genes and T3SS-mediated cytotoxicity in *P.*

aeruginosa (Deng *et al.*, 2017), and was recently deemed to contribute to ciprofloxacin resistance (Long *et al.*, 2020).

2.6.4.1.8. Pail

The highly conserved sRNA Pail (*Pseudomonas anaerobically induced RNA I*) was originally identified as candidate sRNA PA14_13970.1, located in the intergenic region between the genes PA14_13970 and PA14_13990 in *P. aeruginosa* strain PA14 (Tata *et al.*, 2017). It was found to be abundant under anoxic conditions, and rapidly degraded upon a shift to aerobic conditions (Tata *et al.*, 2017). Pail transcription is induced during anaerobic denitrification, and is governed by the nitrate-responsive two-component system NarXL (Tata *et al.*, 2017), whose expression in turn is under the control of the anaerobic regulator Anr (Schreiber *et al.*, 2007). Further studies revealed that Pail is necessary for anaerobic growth of *P. aeruginosa* on glucose in the presence of nitrate (Tata *et al.*, 2017). Pail was also demonstrated to impact the conversion of nitrite to nitric oxide during denitrification.

However, the impact on denitrification seems to be a result of indirect effect(s) on the activity of the nitrite reductase NirS (Tata *et al.*, 2017). In addition, the absence of Pail resulted in impaired growth of the mutant in an *in vivo* CF lung infection model, highlighting the potential importance of Pail for adaptation of *P. aeruginosa* under these conditions (Tata *et al.*, 2017).

2.6.4.1.9. NrsZ

NrsZ represents the first reported nitrogen-regulated sRNA in *P. aeruginosa*, which is highly conserved among *Pseudomonads* (Wenner *et al.*, 2014). It is transcribed from the *ntrC*-PA5126 intergenic region under nitrogen-limiting conditions by the joint action of the nitrogen-responsive two-component system NtrB/C and the sigma factor RpoN (σ^{54} , σ^N) (Wenner *et al.*, 2014). Although the NrsZ full-length transcript is ≈ 226 nt long, the most abundant detected species are ≈ 40 nt and 140 nt in length (Wenner

et al., 2014). Moreover, a processed transcript, encompassing the first 60 nt, was shown to be functional in riboregulation (Wenner *et al.*, 2014). Thus, processing of NrsZ RNA is probably essential for its activity, as it is the case with some other sRNAs such as ArcZ (Papenfort *et al.*, 2009) and MicX (Davis and Waldor, 2007). In *P. aeruginosa*, NrsZ promotes swarming motility by translational activation of the *rhIA* gene, the product of which is required for rhamnolipids biosynthesis (Wenner *et al.*, 2014). Rhamnolipid biosurfactants, together with flagella, are necessary for swarming of *P. aeruginosa* (Caiazza *et al.*, 2005). NrsZ appears to interact with the self-inhibitory secondary structure in the *rhIA* 5'UTR comprising the rbs (Wenner *et al.*, 2014). This interaction melts the intramolecular inhibitory secondary structure and thereby promotes translation of *rhIA* mRNA (Wenner *et al.*, 2014).

2.6.4.2. Dual-function RNA in *P. aeruginosa*

2.6.4.2.1. PhrS

The dual-function RNA PhrS represents the first described anaerobically controlled sRNA in *P. aeruginosa* (Sonnleitner *et al.*, 2011). PhrS synthesis is induced under oxygen-limiting conditions (Sonnleitner *et al.*, 2008), and requires the anaerobic regulator Anr (Sonnleitner *et al.*, 2011). PhrS expression is also affected by Hfq, most probably *via* Hfq-mediated regulation of Anr (Sonnleitner *et al.*, 2011). PhrS was shown to stimulate pyocyanin biosynthesis by increasing the production of PQS (Sonnleitner *et al.*, 2011). PhrS regulates the synthesis of PQS by indirectly activating the translation of *pqsR* mRNA, encoding the QS regulator PqsR (MvfR) (Sonnleitner *et al.*, 2011; Figure 19). PqsR is one of the main regulators of QS in *P. aeruginosa*, which acts as an activator of the *pqsABCDE* operon, required for biosynthesis of PQS (Déziel *et al.*, 2005). The mechanism of PhrS-dependent translational activation of the *pqsR* transcript includes translational coupling, whereby the translation of *pqsR* mRNA is positively coupled with the translation of an *uof*, whose translation in turn is positively regulated by PhrS (Sonnleitner *et al.*, 2011). PhrS base-pairs and resolves the secondary inhibitory

structure occluding the rbs of *uof* and thereby, activates the translation of *uof* and thus, of the *pqsR* mRNA (Sonnleitner *et al.*, 2011; Figure 19).

Fernandez *et al.* (2016) provided evidence for the role of PhrS in the regulation of swarming and biofilm formation in *P. aeruginosa*. The authors observed enhanced swarming and increased biofilm biomass when the levels of PhrS were elevated. The increased levels of PhrS were in turn reconciled with elevated Hfq levels due to decreased expression of its negative regulator Lon under the tested conditions (Fernandez *et al.*, 2016).

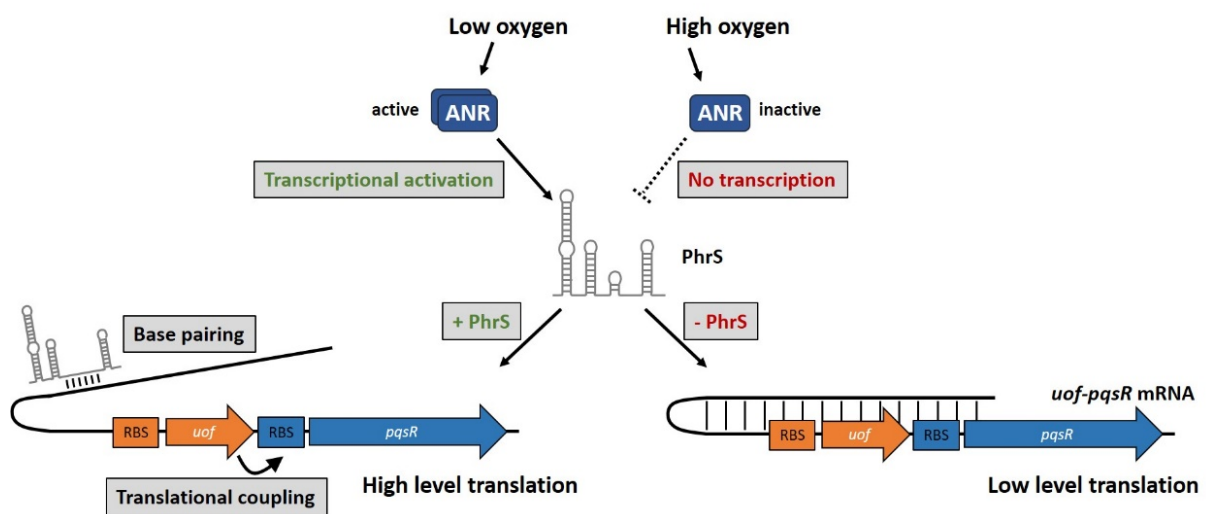


Figure 19. PhrS-dependent translational activation of *pqsR* mRNA. The rbs of *pqsR* mRNA (in blue) and that of the upstream open reading frame (*uof*; in orange) are occluded by a secondary inhibitory structure, which blocks ribosome binding (lower right panel). The sRNA PhrS base-pairs upstream of the rbs of *uof* and resolves the inhibitory stem loop. This in turn leads to *uof-pqsR* translation (lower left panel). Under aerobic conditions, the Phrs levels are low, thus resulting in diminished *uof-pqsR* translation, which consequently leads to moderate levels of PQS (Sonnleitner *et al.*, 2011). Upon oxygen depletion, the anaerobic regulator Anr (dark blue squares) is activated which in turn activates PhrS transcription (upper left panel). High levels of PhrS promote *uof-pqsR* translation, and result in elevated levels of PQS. Figure adapted from Sonnleitner *et al.* (2011).

A recent study proposed a novel function for PhrS as a positive regulator of CRISPR-Cas immunity in *P. aeruginosa* (Lin *et al.*, 2019). Hereby, PhrS was deemed to act as a transcriptional anti-

terminator of Rho-dependent termination by binding to the CRISPR leaders (Lin *et al.*, 2019). This in turn leads to the transcription of the CRISPR locus and synthesis of CRISPR RNA (crRNA), and thus to adaptive immunity (Lin *et al.*, 2019). Apart from its role as a regulatory RNA, PhrS encodes a highly conserved peptide, whose function is still unknown (Sonnleitner *et al.*, 2011).

2.6.4.3. Protein-binding RNAs in *P. aeruginosa*

2.6.4.3.1. RsmV/W/Y/Z

The distinct traits of acute and chronic infections of *P. aeruginosa* are governed by the Rsm post-transcriptional regulatory network (Brencic and Lory, 2009; Goodman *et al.*, 2009; Irie *et al.*, 2010; Sonnleitner *et al.*, 2012a; Romero *et al.*, 2018; Gebhardt *et al.*, 2020; Chihara *et al.*, 2021; Figure 7). The Rsm network of *P. aeruginosa* employs two RNA-binding proteins belonging to the CsrA/RsmA family of proteins, RsmA (Pessi *et al.*, 2001) and RsmN/F (Marden *et al.*, 2013; Morris *et al.*, 2013), as well as four RsmA- and RsmN/F-titrating RNAs, RsmZ/B (Heurlier *et al.*, 2004; Burrowes *et al.*, 2005), RsmY (Valverde *et al.*, 2003; Sonnleitner *et al.*, 2006), RsmW (Miller *et al.*, 2016) and RsmV (Janssen *et al.*, 2018a).

The Rsm RNAs contain multiple unpaired GGA motifs, which serve as Rsm protein binding sites, and act to titrate Rsm proteins from their target mRNAs (Valverde *et al.*, 2003; Heeb *et al.*, 2004; Heurlier *et al.*, 2004; Kay *et al.*, 2006; Lapouge *et al.*, 2008; Miller *et al.*, 2016; Janssen *et al.*, 2018a, 2018b; Figures 7, 17B and 20). Different binding affinities of Rsm proteins for Rsm RNAs are deemed to provide a mechanism of modulating the Rsm regulatory network in response to environmental signals (Janssen *et al.*, 2018b).

The sequestration of the Rsm proteins by the Rsm RNAs leads to the transition from a planktonic /acute infection phenotype to a biofilm /chronic infection phenotype. At the molecular level, the transition results in differential expression of various genes including those encoding

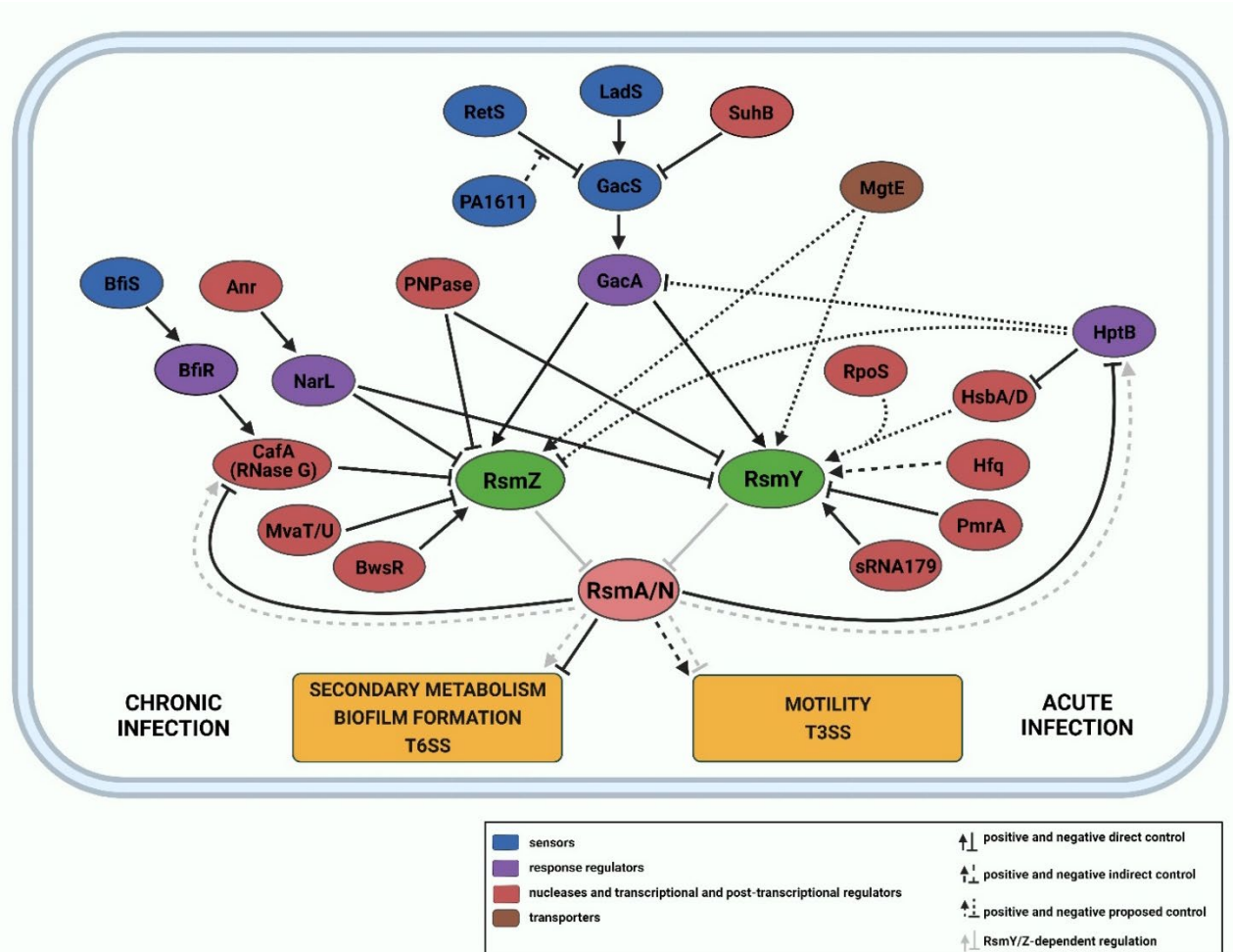


Figure 20. Synthesis and turnover of the RsmY/Z RNAs in *P. aeruginosa*. The RsmY and RsmZ RNAs (green ovals) serve as molecular decoys for the RsmA/N proteins (red oval), antagonizing their regulatory functions, which in turn mediates the transition from an acute to chronic infection (see Figure 7). The transcription and turnover of RsmZ and RsmY are under control of several two-component systems (sensors: blue ovals; response-regulators: violet ovals), transporters (brown ovals), nucleases and transcriptional and post-transcriptional regulators (dark red ovals), which act either in a GacA/S-dependent- (*i.e.* SuhB-mediated repression of RsmY/Z synthesis) or independent manner (*i.e.* Anr-mediated repression of RsmY/Z expression). Some regulatory pathways modulate the abundance of both RNAs (*e.g.* GacA/S transcriptional activation of RsmY/Z), whereas some are specific for only one of the RNAs (*e.g.* BfiR-dependent CafA degradation of RsmZ and Hfq-dependent stabilization of RsmY). The complex regulation of the Rsm RNAs enables fine-tuning of the expression of RsmA/N targets in response to different environmental cues. ↓: positive control; ⊥: negative control; dashed lines: indirect control; dotted lines: proposed mechanism. Light grey lines indicate RsmY/Z-dependent regulation. Figure adapted from Sonnleitner *et al.* (2012a).

components of the T3SS and the T6SS, respectively (Mulcahy *et al.*, 2006; Burrowes *et al.*, 2006; Lapouge *et al.*, 2008; Brencic and Lory, 2009; Brencic *et al.*, 2009; Bordi *et al.*, 2010; Irie *et al.*, 2010; Sonnleitner *et al.*, 2012a; Marden *et al.*, 2013; Intile *et al.*, 2014; Miller *et al.*, 2016; Li *et al.*, 2017; Romero *et al.*, 2018; Janssen *et al.*, 2018a, 2018b; Gebhardt *et al.*, 2020; Chihara *et al.*, 2021; Figures 7 and 20).

RsmY and RsmZ are the main RsmA/RsmN/F binding RNAs (Lapouge *et al.*, 2008; Brencic and Lory, 2009; Janssen *et al.*, 2018b), while RsmV and RsmW seems to contribute to the dosage effect and temporal modulation of the RsmA/RsmN/F regulatory cascade in response to specific environmental signals in *P. aeruginosa* strain PAO1 (Miller *et al.*, 2016; Janssen *et al.*, 2018a, 2018b). Each Rsm RNA displays different and unique expressions patterns and mechanisms for turnover and stability. For instance, the synthesis of RsmY and RsmZ is transiently elevated in late logarithmic growth phase, and then significantly increases in late stationary phase, whereas the onset of *rsmW* transcription was observed in early stationary phase (Janssen *et al.*, 2018a). On the contrary, the steady-state levels of RsmV are steadily increasing throughout growth (Janssen *et al.*, 2018a). Moreover, *rsmY*, *rsmZ* and *rsmW* expression was found to be elevated in biofilms (Petrova and Sauer, 2010; Miller *et al.*, 2016). However, RsmZ appears to repress early biofilm formation (Kay *et al.*, 2006), highlighting the importance of controlled fine-tuning of the Rsm RNA levels (Petrova and Sauer, 2010).

The differential expression patterns of the Rsm RNAs can be attributed to distinct mechanisms of regulation of their transcription and turnover. Whereas the transcription of *rsmY* and *rsmZ* is directly controlled by the two-component system GacA/S (Kay *et al.*, 2006; Brencic *et al.*, 2009; Figure 20) and by the two orphan sensor kinases, LadS and RetS (Goodman *et al.*, 2004; Ventre *et al.*, 2006; Figure 20), the expression of *rsmW* and *rsmV* does not directly depend on the GacA/S-system (Miller *et al.*, 2016; Janssen *et al.*, 2018a).

The GacA/S (*global activation of antibiotic and cyanide synthesis*) two-component system regulates a variety of different processes in bacteria ranging from primary and secondary metabolism

to virulence. It is conserved in *Pseudomonads* (Hrabak and Willis, 1992; Laville *et al.*, 1992; Reimann *et al.*, 1997; Chancey *et al.*, 1999; Willis *et al.*, 2001; Chatterjee *et al.*, 2003; Zuber *et al.*, 2003; Lapouge *et al.*, 2008; Goodman *et al.*, 2009) and widely present in other gamma-proteobacteria (Castañeda *et al.*, 2001; Cui *et al.*, 2001; Pernestig *et al.*, 2001; Hammer *et al.*, 2002; Suzuki *et al.*, 2002; Whistler and Ruby, 2003). A yet unidentified signal triggers auto-phosphorylation and the activation of the sensor kinase GacS, which in turn phosphorylates the response regulator GacA. Phosphorylated GacA binds to conserved GacA-boxes, also known as *upstream activating sequence* (UAS) in the promoter region of target genes and activates their transcription (Humair *et al.*, 2010). The activity of GacS is under the control of RetS and LadS (Goodman *et al.*, 2004; Laskowski *et al.*, 2004; Laskowski and Kazmierczak, 2006; Ventre *et al.*, 2006). The sensor kinase RetS directly binds to GacS and impedes its auto-phosphorylation, and thus prevents subsequent activation of the response regulator GacA (Goodman *et al.*, 2009). In this way, RetS activates T3SS, represses biofilm formation, and promotes colonization/dissemination in acute infection murine models (Goodman *et al.*, 2004; Laskowski *et al.*, 2004; Zolfaghar *et al.*, 2005). In contrast, the orphan kinase LadS activates the response regulator GacA by phosphorylation (Chambonnier *et al.*, 2016). Thus, RetS and LadS are antagonistically driving the expression of GacA/S-dependent genes, enabling the transition between acute and chronic infection phenotypes (Ventre *et al.*, 2006). Further study reported that the hybrid sensor kinase PA1611 binds to RetS and interferes with its binding to GacS, and thus indirectly alters the synthesis of RsmY and RsmZ RNAs (Bhagirath *et al.*, 2017). Another hybrid sensor kinase, HptB, was shown to negatively control *rsmY* expression in the planktonic cells in a GacA/S-dependent manner (Bordi *et al.*, 2010; Jean-Pierre *et al.*, 2017). Recently, it was demonstrated that during stationary phase HptB can also alter the synthesis of RsmY independently of the GacA/S regulatory cascade *via* an HsbA/D and RpoS-mediated pathway (Bouillet *et al.*, 2019). Moreover, HptB also inhibits *rsmZ* transcription in a GacA/S-independent way in swarming cells (Jean-Pierre *et al.*, 2017; Figure 20). Similarly, the ribosome-associated protein SuhB, which is deemed to promote synthesis of T3SS and to inhibit

synthesis of T66SS, indirectly inhibits the production of RsmY and RsmZ by repressing *gacA* expression (Li *et al.*, 2013a; Figure 20).

Further studies have shown that the magnesium transporter MgtE, which acts as a negative regulator of T3SS, also affects *rsmY* and *rsmZ* expression (Chakravarty *et al.*, 2017; Figure 20). In contrast, Anr-dependent activation of T3SS under anoxic conditions appears to be mediated by the transcription factor NarL, which acts as a direct transcriptional repressor of *rsmY* and *rsmZ* (O'Callaghan *et al.*, 2011; Figure 20). Furthermore, the histone-like nucleoid structuring proteins MvaT and MvaU, were previously demonstrated to inhibit *rsmZ* transcription by binding to AT-rich region upstream of the *rsmZ* promoter (Castang *et al.*, 2008; Brencic *et al.*, 2009; Figure 20). The inhibitory effects of MvaT/MvaU are in turn counteracted by the transcriptional regulator BswR (Wang *et al.*, 2014a; Figure 20). Moreover, the sRNA179 was found to modulate the Rsm system by promoting transcription of *rsmY*, which results in the inhibition of expression of the cAMP-Vfr and the T3SS regulons (Janssen *et al.*, 2020; Figure 20). However, no effect on RsmZ production was observed. On the contrary, the response regulator PmrA binds directly to the promoter region of *rsmY*, but not to that of *rsmZ*, and inhibits *rsmY* expression (Liu *et al.*, 2018; Figure 20).

In contrast to *rsmY* and *rsmZ* transcription, neither RsmW, nor RsmV synthesis is activated by the GacA/S system (Miller *et al.*, 2016; Janssen *et al.*, 2018a). RsmW seems to be derived from the 3'UTR of the upstream gene PA4570, a protein of unknown function (Miller *et al.*, 2016). *In silico* studies of the *rsmV* promoter region suggested the transcriptional activators RhIR, AlgU, and FleQ as potential regulators of RsmV synthesis (Janssen *et al.*, 2018a). However, subsequent functional studies did not provide any further support for these observations (Janssen *et al.*, 2018a).

Previous studies have shown that RsmA stabilizes and protects the RsmY and RsmZ RNAs from nucleolytic decay (Reimmann *et al.*, 2005; Kay *et al.*, 2006). Furthermore, RsmA and Hfq can interact with RsmY simultaneously (Sorger-Domenigg *et al.*, 2007). Hfq binds to and stabilizes the RsmY transcript (Sonnleitner *et al.*, 2006; Figure 20) by preventing RNase E-dependent degradation (Sorger-

Domenigg *et al.*, 2007). Moreover, Hfq seems also to stabilize RsmW RNA (Miller *et al.*, 2016). However, Hfq does not bind to and it is not implicated in the RsmZ decay (Sonnleitner *et al.*, 2006). Another study revealed that the two-component system BfiR/S activates ribonuclease RNase G (CafA) during biofilm development, which in turn stimulates RsmZ, but not RsmY turnover (Petrova and Sauer, 2010; Figure 20). In addition, polynucleotide phosphorylase (PNPase), a phosphate-dependent 3'–5' exonuclease previously linked to virulence and bacterial stress response (Rosenzweig and Chopra, 2013), was reported to regulate multiple virulence factors by affecting RsmY and RsmZ turnover (Chen *et al.*, 2016). Chihara *et al.* (2021) recently demonstrated that RsmA and RsmN/F proteins can also alter *rsmZ* and *rsmY* expression indirectly by inhibiting the RNase G (CafA) and the HptB/HsbA/D pathway, respectively (Figure 20).

2.6.4.3.2. CrcZ

In *Pseudomonas*, the hierarchical assimilation of carbon sources is governed by the concerted action of the RNA chaperone Hfq (Sonnleitner and Bläsi, 2014) and the carbon catabolite repression protein Crc (MacGregor *et al.*, 1991; Hester *et al.*, 2000a, 2000b; Sonnleitner *et al.*, 2009, 2012b; Linares *et al.*, 2010; La Rosa *et al.*, 2015, 2016; Reales-Calderón *et al.*, 2015; Corona *et al.*, 2018; Molina *et al.*, 2019). Hfq/Crc-mediated regulation is antagonized by the protein-binding RNA CrcZ (Sonnleitner *et al.*, 2009, 2018; Sonnleitner and Bläsi, 2014). CrcZ RNA is highly conserved in the genus *Pseudomonas* (Moreno *et al.*, 2012) and some *Pseudomonas* species contain additional genetically and/or functionally redundant CrcZ RNA homologs such as CrcY and CrcX in *P. syringae* (Filiatrault *et al.*, 2010, 2013), and CrcY in *P. putida* (Moreno *et al.*, 2012) and *P. fluorescens* (Moreno *et al.*, 2012; Liu *et al.*, 2017), respectively.

The CrcZ RNA and its homologs carry several A-rich motifs, which were shown to interact with the distal RNA binding surface of Hfq (Sonnleitner *et al.*, 2009; Sonnleitner and Bläsi, 2014; Moreno *et*

al., 2015; Liu *et al.*, 2017; Chihara *et al.*, 2019). For instance, Hfq has a ~ 5-20-fold higher affinity for CrcZ/Y than for its target *amiE* mRNA (Sonnleitner and Bläsi, 2014; Liu *et al.*, 2017; Sonnleitner *et al.*, 2017). Hence, the sequestration of Hfq by CrcZ/Y seems to prevent Hfq from binding to target mRNAs and usually leads to de-repression of their translation (Sonnleitner and Bläsi, 2014; La Rosa *et al.*, 2015; Moreno *et al.*, 2015; Liu *et al.*, 2017; Sonnleitner *et al.*, 2018).

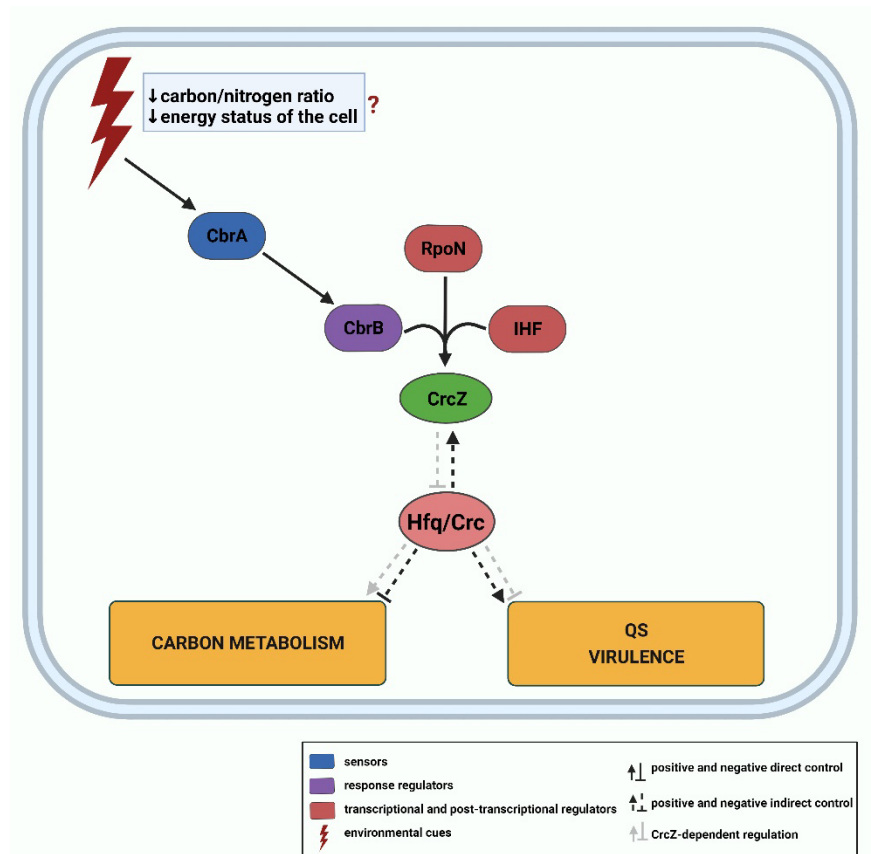


Figure 21. Regulation of the transcription and stability of the CrcZ RNA in *P. aeruginosa*. The CrcZ RNA (green oval) competes for Hfq and/or Hfq/Crc repressive complexes (red oval) with their target mRNAs, and thereby cross-regulates physiological processes involved in carbon metabolism and virulence (light grey lines; see Figure 15). The synthesis of CrcZ RNA occurs under energy-limiting conditions in the presence of an unfavourable carbon source by the joint action of the CbrA/B two-component system (blue and violet oval, respectively), IHF and the sigma factor RpoN (dark red ovals), which are activated by a yet unknown trigger (red question mark). Moreover, CrcZ is stabilized by Hfq and/or Hfq/Crc. ↓: positive control; ⊥: negative control; dashed lines: indirect control.

In *P. aeruginosa*, the 407 nt long CrcZ RNA is encoded in the *cbrB-pcnB* intergenic region (Sonnleitner *et al.*, 2009). The *crcZ* gene is transcribed from an RpoN-dependent promoter with the aid of the response regulator CbrB and integration host factor (IHF) (Sonnleitner *et al.*, 2009; Abdou *et al.*, 2011; Figure 21). CbrB together with its cognate histidine kinase CbrA forms a TCS involved in the maintenance of carbon and nitrogen balance (Nishijyo *et al.*, 2001; Li and Lu, 2007). CbrA/B is activated by an unknown signal which might depend on the energy status of the cell (Valentini *et al.*, 2014). Moreover, the CbrA/B activity and the resulting CrcZ levels depend on a given carbon source (Sonnleitner *et al.*, 2009; Valentini *et al.*, 2014). For instance, the CrcZ levels are low during fast growth

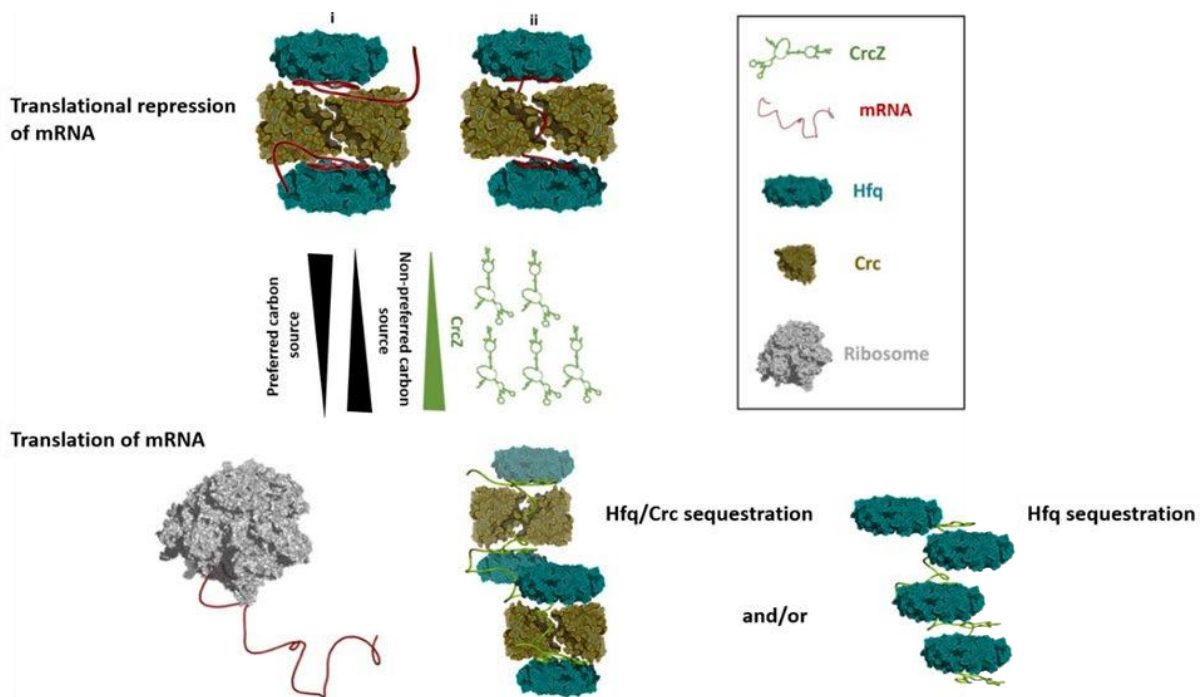


Figure 22. Model for the CrcZ/Hfq/Crc-mediated regulation of CCR in *Pseudomonas aeruginosa*. During growth in the presence of a preferred carbon source, *e.g.* succinate (upper panel), the levels of the regulatory RNA CrcZ (in green) are low and the RNA chaperone Hfq (in cyan) and its co-repressor, catabolite repression protein Crc (in brown) form a repressive ribonucleoprotein complex on target mRNAs (in red), translation of which is repressed during CCR. Upon the depletion of the preferred substrate (lower panel), the transcription of CrcZ RNA is elevated, and CrcZ acts as a molecular decoy for Hfq by binding to its distal site. This in turn, results in a relief of CCR as Hfq and /or the Hfq/Crc repressive complex is sequestered by CrcZ (lower right panel) and the repression of target mRNAs is alleviated (lower left panel), enabling the utilization and growth on less preferred carbon sources. Figure adapted from Pei *et al.* (2019).

in the presence of a preferred carbon source, *e.g.* succinate (Sonnleitner *et al.*, 2009; Valentini *et al.*, 2014). In contrast, CrcZ is highly abundant during slow growth in the presence of non-favourable carbon sources, *e.g.* oxaloacetate (Valentini *et al.*, 2014). Hernandez-Arranz *et al.* (2016) demonstrated that the *crcZ* expression can also be driven from the weak constitutive promoter of the *cbrB* gene resulting in a *cbrB-crcZ* transcript, which is subsequently processed to yield the ≈ 407 nt long CrcZ RNA. In addition, several shorter variants of CrcZ seem to result from either premature termination of transcription or from degradation by a yet unknown nuclease (Sonnleitner *et al.*, 2009). Previously, it was shown that Hfq in concert with Crc affects both the transcription and stability of CrcZ in *P. putida* (Hernández-Arranz *et al.*, 2016).

It has been demonstrated that both, Hfq and Crc, are necessary for tight translational repression of mRNAs which are under the control of CCR (Sonnleitner and Bläsi, 2014; Moreno *et al.*, 2015). The protein levels of Hfq and Crc appear to remain constant under different growth conditions (Sonnleitner and Bläsi, 2014). Thus, the response to distinct carbon sources is mediated by different levels of the protein-titrating RNA CrcZ/Y (Sonnleitner and Bläsi, 2014; Moreno *et al.*, 2015; Liu *et al.*, 2017; Sonnleitner *et al.*, 2018; Figures 21 and 22). In addition, through the competition for Hfq, CrcZ can also indirectly cross-regulate other physiologically important processes which are subject to Hfq-mediated regulation (Sonnleitner *et al.*, 2017).

3. Aims of the work

The overall goal of this work was to study the involvement of the two key regulators of CCR in *P. aeruginosa*, the global post-transcriptional regulator Hfq and the Hfq-binding RNA CrcZ in biofilm formation and antibiotic susceptibility.

The first aim was to identify and to assess the Hfq-RNA-interactome and the transcriptome during anoxic biofilm formation.

The second aim was to identify key regulators governing the synthesis of the transcriptional activator of anaerobic metabolism Anr.

The third aim was to study the role of Hfq in susceptibility of *P. aeruginosa* PAO1 and PA14 toward different classes of antibiotics, and to test whether the Hfq-titrating RNA CrcZ can be harnessed to modulate Hfq-mediated antibiotic susceptibility.

The fourth aim was to dissect the Hfq-mediated regulatory mechanisms governing *oprD* and *opdP* expression, encoding porins that serve as entry channels for carbapenem antibiotics.

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5.1 Cross-regulation by CrcZ RNA controls anoxic biofilm formation in *Pseudomonas aeruginosa*

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Cross-regulation by CrcZ RNA controls anoxic biofilm formation in *Pseudomonas aeruginosa*

Petra Pusic¹, Muralidhar Tata¹, Michael T. Wolfinger^{2,3}, Elisabeth Sonnleitner¹, Susanne Häussler⁴ & Udo Bläsi¹

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Pseudomonas aeruginosa (PA) can thrive in anaerobic biofilms in the lungs of cystic fibrosis (CF) patients. Here, we show that CrcZ is the most abundant PA14 RNA bound to the global regulator Hfq in anoxic biofilms grown in cystic fibrosis sputum medium. Hfq was crucial for anoxic biofilm formation. This observation complied with an RNAseq based transcriptome analysis and follow up studies that implicated Hfq in regulation of a central step preceding denitrification. CrcZ is known to act as a decoy that sequesters Hfq during relief of carbon catabolite repression, which in turn alleviates Hfq-mediated translational repression of catabolic genes. We therefore inferred that CrcZ indirectly impacts on biofilm formation by competing for Hfq. This hypothesis was supported by the findings that over-production of CrcZ mirrored the biofilm phenotype of the *hfq* deletion mutant, and that deletion of the *crcZ* gene augmented biofilm formation. To our knowledge, this is the first example where competition for Hfq by CrcZ cross-regulates an Hfq-dependent physiological process unrelated to carbon metabolism.

PA can form persistent biofilms in the lungs of CF patients¹. Polymorphonuclear leukocytes are known to surround the biofilms in the CF lung and to consume the majority of O₂ to produce reactive oxygen species, which suggested that PA biofilms may partially grow anaerobically in this environment^{2,3}. Based on several studies, Yoon *et al.*⁴ provided first hints for anaerobic respiration in PAO1 biofilms. This has been recently supported through the quantification of compounds of the denitrification pathway in sputum of CF patients⁵.

In *Enterobacteriaceae*, the RNA chaperone Hfq is known to play a key role in riboregulation by facilitating the interaction between small regulatory RNAs (sRNAs) and their target mRNAs⁶. As a number of sRNAs have been shown to regulate virulence traits including biofilm formation in different bacterial pathogens⁷, the requirement for Hfq in RNA-mediated regulation most likely contributes to the observed attenuated virulence phenotype of respective *hfq* mutants⁶. In PAO1, a deletion of the *hfq* gene resulted in pleiotropic effects on growth and virulence⁸.

Although many putative regulatory RNAs have been identified in different PA strains⁹, the function of only a few has been revealed, and only three regulatory RNAs have been implicated in biofilm formation. The sRNA PhrS represents the founding member of anaerobically controlled PA sRNAs¹⁰. PhrS has been suggested to be involved in biofilm formation as it was shown to stimulate the synthesis of the *Pseudomonas* quinolone signal (PQS)¹⁰, which can induce the release of DNA that serves as a biofilm matrix component¹¹. A stimulatory effect of PhrS on biofilm formation has been recently observed¹². The PA protein binding RNAs RsmY and RsmZ antagonize the function of the translational regulator RsmA¹³. The RsmA protein is known to act as a translational repressor of *psl* mRNA, which prevents exopolysaccharide synthesis, and thus to control biofilm formation in a negative manner¹⁴. On the other hand, up-regulation of the RsmY/Z RNAs results in titration of RsmA, and therefore in increased biofilm formation^{7,13}.

The aim of this study was to identify PA14 regulatory RNAs involved in anoxic biofilm formation, which is a poorly studied aspect of chronic PA infections. We show that the Hfq-binding RNA CrcZ is highly abundant under these conditions, and that it impacts on anoxic biofilm formation. Thus, in addition to its established role

¹Department of Microbiology, Immunobiology and Genetics, Center of Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter, Dr. Bohr-Gasse 9, 1030 Vienna, Austria. ²Institute of Theoretical Chemistry, University of Vienna, Währingerstrasse 17, 1090 Vienna, Austria. ³Center for Anatomy and Cell Biology, Medical University of Vienna, Währingerstrasse 13, 1090 Vienna, Austria. ⁴Helmholtz Center for Infection Research, Molecular Bacteriology, Inhoffenstraße 7, 38124 Braunschweig, Germany. Correspondence and requests for materials should be addressed to U.B. (email: udo.blaesi@univie.ac.at)

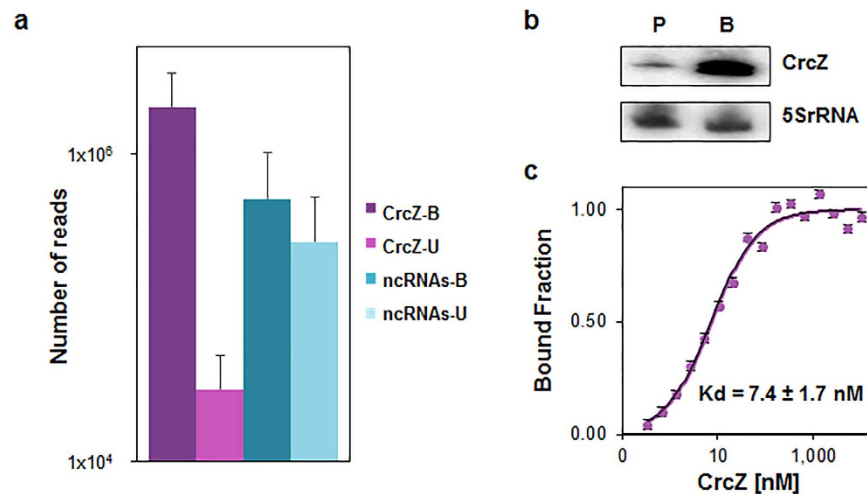


Figure 1. CrcZ is the major regulatory RNA bound to Hfq in anoxic biofilms. (a) Hfq-bound RNAs were isolated from lysates of B-96 cultures after co-immunoprecipitation with Hfq-specific antibodies. The identity of Hfq-bound and unbound RNAs in the supernatant was revealed by RNAseq. The number of reads for CrcZ in the Hfq-bound fraction (CrcZ-B; dark purple bar) and unbound fraction (CrcZ-U; light purple bar) is shown in comparison to the number of reads for all other described or putative PA14 regulatory RNAs in the Hfq-bound (ncRNAs-B; blue bar) and unbound fraction (ncRNAs-U; light blue bar). Error bars, mean $\pm$ s.d. of 2 biological replicates. (b) The steady state levels of CrcZ are elevated in B-96 cultures. The PA14 strain was grown in SCFM under aerobic conditions to an OD₆₀₀ of 2 (P) and for 96 hours in anoxic biofilms (B), respectively. Total RNA was isolated and 10 μ g of total RNA was used for the Northern-blot analysis. (c) K_d of Hfq for CrcZ RNA revealed by microscale thermophoresis. Increasing amounts of non-labelled *in vitro* transcribed CrcZ were added to 20 nM fluorescently labelled Hfq protein. The dissociation constant (K_d) of CrcZ was determined as described⁴⁷, and was expressed as mean EC50 $\pm$ EC50 confidence interval of 2 independent experiments.

in carbon catabolite repression, where CrcZ acts as a decoy to abrogate Hfq-mediated translational repression of catabolic genes¹⁵, this study reveals a novel aspect of Hfq sequestration by CrcZ, that is cross-regulation of other Hfq-dependent physiological processes.

Results and Discussion

With the goal to identify regulatory RNAs that impact on anoxic biofilm formation, we concentrated on RNAs that interact with Hfq. The PA14 strain was grown in modified cystic fibrosis sputum medium (SCFM)¹⁶, which approximates to the conditions of the CF lung. Upon anaerobic biofilm growth of PA14 for 96 h in SCFM (B-96 cultures), Hfq-bound RNAs were isolated by co-immunoprecipitation with Hfq-specific antibodies. The identity of Hfq-bound and unbound putative and confirmed regulatory RNAs of PA14¹⁷ was revealed by RNAseq (Supplementary Table S1). All other reads were excluded from further analyses. Based on the total reads obtained for these RNAs (Hfq-bound and unbound), CrcZ was the most abundant PA14 regulatory RNA in B-96 cultures (Fig. 1a). The RNAseq results were mirrored by a Northern-blot analysis, showing that the levels of CrcZ RNA were ~50-fold higher in B-96 cultures than in planktonically grown PA14 cultures (OD₆₀₀ = 2.0; P) (Fig. 1b). Moreover, the reads obtained for Hfq-bound CrcZ RNA outnumbered all other described or putative PA14 regulatory RNAs that interacted with Hfq by a factor of 4 (Fig. 1a). For verification, the Hfq-bound and unbound fractions were tested by RT-PCR for the presence of CrcZ RNA, ErsA RNA, which requires Hfq for function¹⁸, and RsmZ RNA, which poorly binds to Hfq¹⁹. Both, CrcZ and ErsA, were found in complex with Hfq, whereas the majority of RsmZ RNA was detected in the unbound fraction (Supplementary Fig. S1).

CrcZ transcription requires the alternative sigma factor RpoN and the response regulator CbrB, which is phosphorylated by the sensor/histidine kinase CbrA^{20,21}. A recent transcriptome analysis indicated that the *cbrA* gene is 2.5-fold up-regulated in B-96 cultures when compared to P cultures²². This observation may partially rationalize the up-regulation of CrcZ in B-96 cultures. On the other hand, a putative motif for the anaerobic regulator Anr was identified upstream of the RpoN-dependent *crcZ* promoter (Supplementary Fig. S2). As *crcZ* was poorly expressed in a PA14 *anr*- strain (Supplementary Fig. S2), it remains to be shown whether the observed up-regulation of *crcZ* in B-96 cultures (Fig. 1b) is indeed mediated by Anr.

As the intracellular quantities of a given Hfq-bound RNA depend on its abundance as well as on its affinity for the protein, we next determined the K_d of Hfq for CrcZ using microscale thermophoresis. The K_d was determined with ~7.4 nM (Fig. 1c). The high K_d of Hfq for CrcZ can be reconciled with six A-rich stretches in CrcZ^{15,20} to which Hfq can bind with its distal tripartite binding motifs^{15,23}. The majority of Hfq was co-immunoprecipitated from the lysate of B-96 cultures (Supplementary Fig. S3) and CrcZ was the most abundant Hfq-binding RNA (Fig. 1a). Therefore, we next asked whether Hfq is crucial for and whether competition for Hfq by CrcZ can interfere with anoxic growth and biofilm formation.

To test whether Hfq is crucial for anaerobiosis, the growth of PA14 and PA14 Δ hfq was monitored in SCFM. Under these conditions growth of the PA14 Δ hfq was impaired (Supplementary Fig. S4). In addition, the metabolic

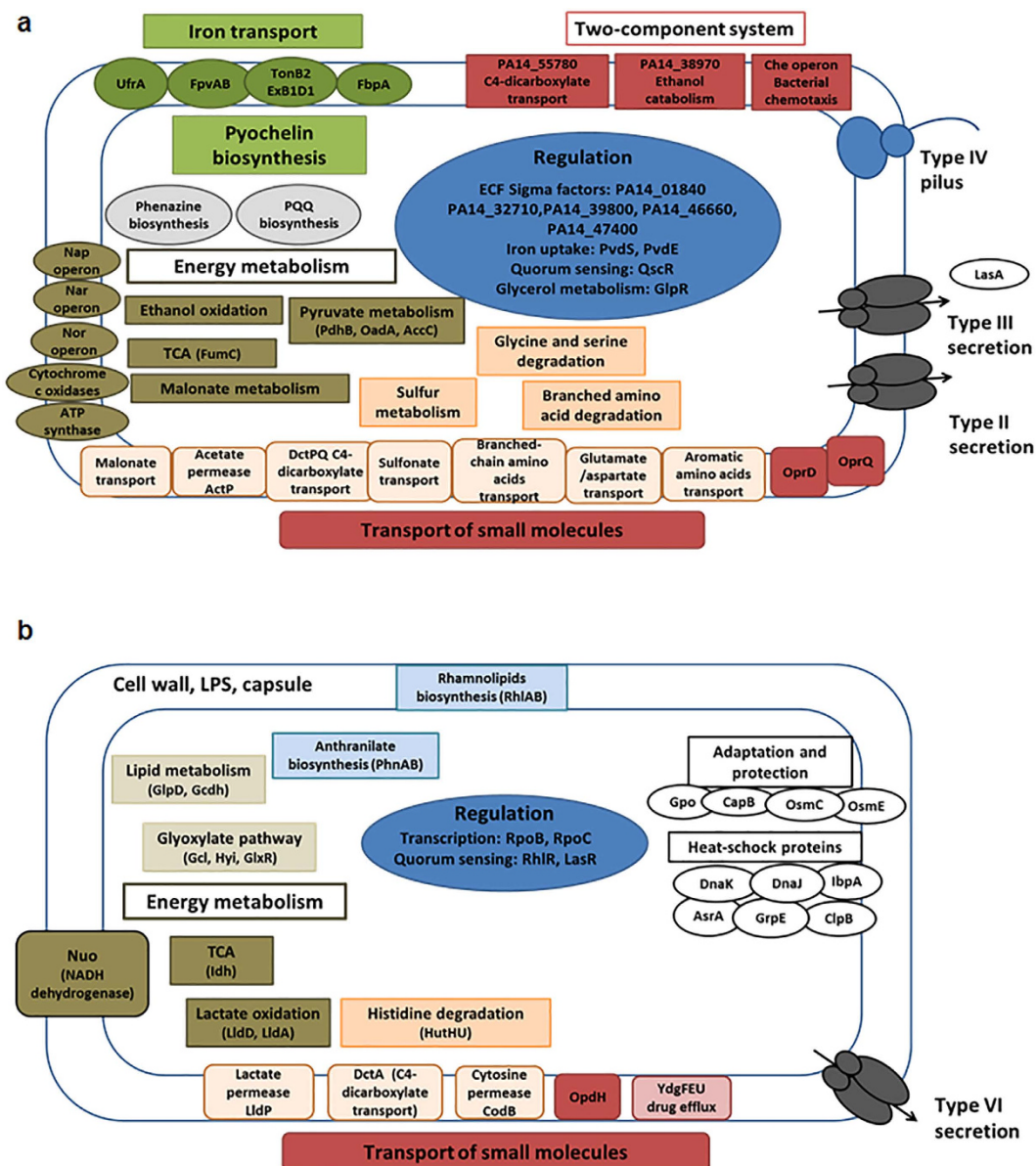


Figure 2. Hfq regulon in B-96 cultures. Regulatory and metabolic networks affected by Hfq. Increased (a) and decreased abundance (b) of the transcripts encoding for the corresponding proteins in PA14Δ*hfq* versus PA14. Functional classification according to the Pseudomonas Genome database⁵² and KEGG (Kyoto Encyclopedia of Genes and Genome)⁵³.

activity of PA14 and PA14Δ*hfq* was assessed during anoxic growth in SCFM by employing a *PrnBP1-gfp* (AGA) reporter gene²⁴. When compared with strain PA14, the absence of Hfq resulted in a decrease of the GFP activity, indicating that PA14Δ*hfq* is less metabolically active than the wild-type strain (Supplementary Fig. S4).

These initial studies prompted us to perform a RNAseq based comparative transcriptome analysis with B-96 cultures of PA14 and the PA14Δ*hfq* mutant, respectively, to assess the impact of Hfq on anoxic biofilm formation in more detail. For the initial analysis a p-value (adjusted for multiple testing) of 0.05 was set as a threshold for statistical significance and the change in abundance (fold change) had to exceed ± 4 for a given transcript in order to be considered differentially abundant. In addition, transcripts were also considered, if they were differentially abundant at least ± 1.5 -fold and part of an affected pathway or operon (Supplementary Table S2). 428 transcripts were found to be differentially expressed in the *hfq* mutant when compared with PA14; 296 and 132 transcripts showed at least a 4-fold increased and decreased abundance, respectively (Supplementary Table S2).

These analyses revealed functions implicated in anoxic biofilm formation, which might be under direct or indirect control of Hfq (Fig. 2, Supplementary Table S2). Among them, the transcript encoding the quorum-sensing regulator QscR, known to act as a negative regulator of LasR and RhlR²⁵, was 9.23-fold up-regulated in the absence of Hfq (Fig. 2a, Supplementary Table S2a). Accordingly, transcripts encoding the quorum-sensing regulators RhlR and LasR, as well as downstream genes involved in rhamnolipid biosynthesis (*rhlAB*) were down-regulated

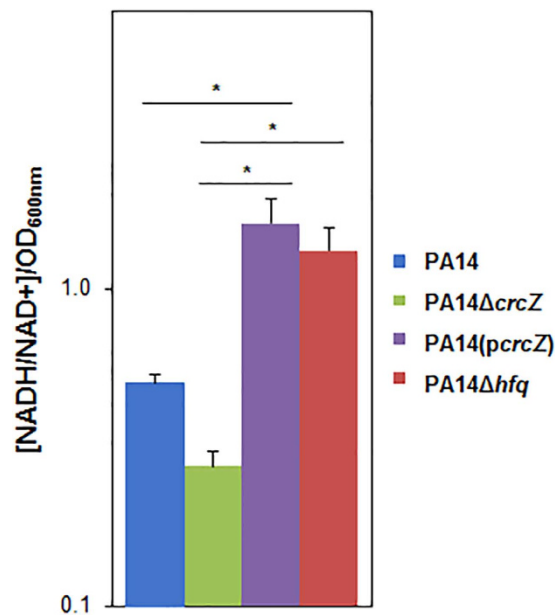


Figure 3. The absence of Hfq causes a redox imbalance. The NADH/NAD⁺ levels were assessed in B-96 cultures of PA14 (blue bar), PA14ΔcrcZ (green bar), PA14(pcrcZ) (violet bar) and PA14Δhfq (red bar) grown in SCFM⁴⁸. Error bars, mean ± s.d. N = 3 biological replicates with 3 technical replicates. **P* < 0.05, analysed by one-way ANOVA with the Tukey's HSD post hoc test.

in the absence of Hfq (Fig. 2b, Supplementary Table S2b). As an impairment of QS can result in killing of PAO1 in anaerobic biofilms⁴, a de-regulation of the *qscR* gene in PA14Δhfq could thus impact on anoxic biofilm formation.

The abundance of the transcript encoding glycerol-3-phosphate dehydrogenase GlpD, which serves as a major link between carbohydrate and lipid metabolism, was 4.84-fold decreased in the absence of Hfq (Fig. 2b, Supplementary Table S2b). This observation can be reconciled with the 11.51-fold increased abundance of the *glpR* transcript encoding the negative regulator of *glpD* (Fig. 2a, Supplementary Table S2a). As glycerol metabolism plays an important role in *P. aeruginosa* persistence by promoting biofilm formation²⁶, a possible de-regulation of *glpD* in PA14Δhfq might also interfere with anoxic biofilm formation.

PA can thrive under anaerobic conditions by acquiring ATP from glycolysis, pyruvate fermentation²⁷, the arginine deiminase pathway²⁸ as well as from denitrification²⁹. When compared with PA14, the absence of Hfq resulted in increased abundance of transcripts encoded by the *nar*, *nap* and *nor* operons, encoding enzymes required for denitrification (Fig. 2a, Supplementary Table S2a). In contrast, the transcripts encoding the nitrite reductase (*nir*-operon) and the nitrous dioxide reductase (*nos* operon) did not show a differential abundance. However, a number of *nuo* transcripts, encoding subunits of the NADH dehydrogenase, were down-regulated in the absence of Hfq (Fig. 2b, Supplementary Table S2b). The NADH dehydrogenase is required for anaerobic growth in the presence of nitrate³⁰. It contributes to the intracellular redox balance, i.e. the NADH/NAD⁺ ratio³¹, and its activity is coupled to the energizing processes of the membrane and ATP synthesis³², which in turn is required for sustained anoxic biofilm formation.

A redox imbalance can impact on the fitness of bacteria³³. As the transcriptome analysis indicated a lower abundance of some *nuo* transcripts as well as of other transcripts encoding dehydrogenases (*glpD*; *lldA*; *lldD*; Supplementary Table S2b) in the absence of Hfq, we next asked whether Hfq affects the intracellular NADH/NAD⁺ ratio. The NADH/NAD⁺ ratios were determined in B-96 cultures of strains PA14, PA14ΔcrcZ, PA14(pcrcZ) and PA14Δhfq, respectively. When compared with strains PA14 and PA14ΔcrcZ, an increase in NADH/NAD⁺ ratio was observed in PA14Δhfq (Fig. 3). As the NADH/NAD⁺ ratio is crucial for the function of metabolic pathways, it seems reasonable to speculate that the reduced metabolic activity of the PA14Δhfq strain (Supplementary Fig. S4) results at least in part from a redox imbalance.

Since the deletion of the *crcZ* gene augmented the metabolic activity of strain PA14ΔcrcZ (Supplementary Fig. S4) and over-expression of a plasmid borne copy of *crcZ* mirrored both the anoxic growth phenotype and the increased NADH/NAD⁺ ratio of the PA14Δhfq strain (Supplementary Fig. S4, Fig. 3), we next examined whether differential CrcZ levels affect anoxic biofilm formation. First, anoxic biofilm formation was assessed by a static crystal violet assay³⁴ in B-96 cultures of the strains PA14, PA14ΔcrcZ, PA14(pcrcZ) and PA14Δhfq, respectively. When compared with PA14, anoxic biofilm formation was increased in PA14ΔcrcZ, whereas ectopic over-production of CrcZ (Supplementary Fig. S5) reduced anoxic biofilm formation to a level comparable with the PA14Δhfq strain (Supplementary Fig. S5).

Second, the viability and biomass of B-96 cultures of PA14, PA14ΔcrcZ, PA14(pcrcZ) and PA14ΔhfqΔcrcZ was assessed by live/death staining and visualized by confocal laser scanning microscopy (Fig. 4). When compared with PA14, a marked increase in both, the total and live cell biomass was observed for PA14ΔcrcZ (Fig. 4). In contrast, when compared to PA14, an increase of dead cells was observed in anoxic biofilms of the CrcZ

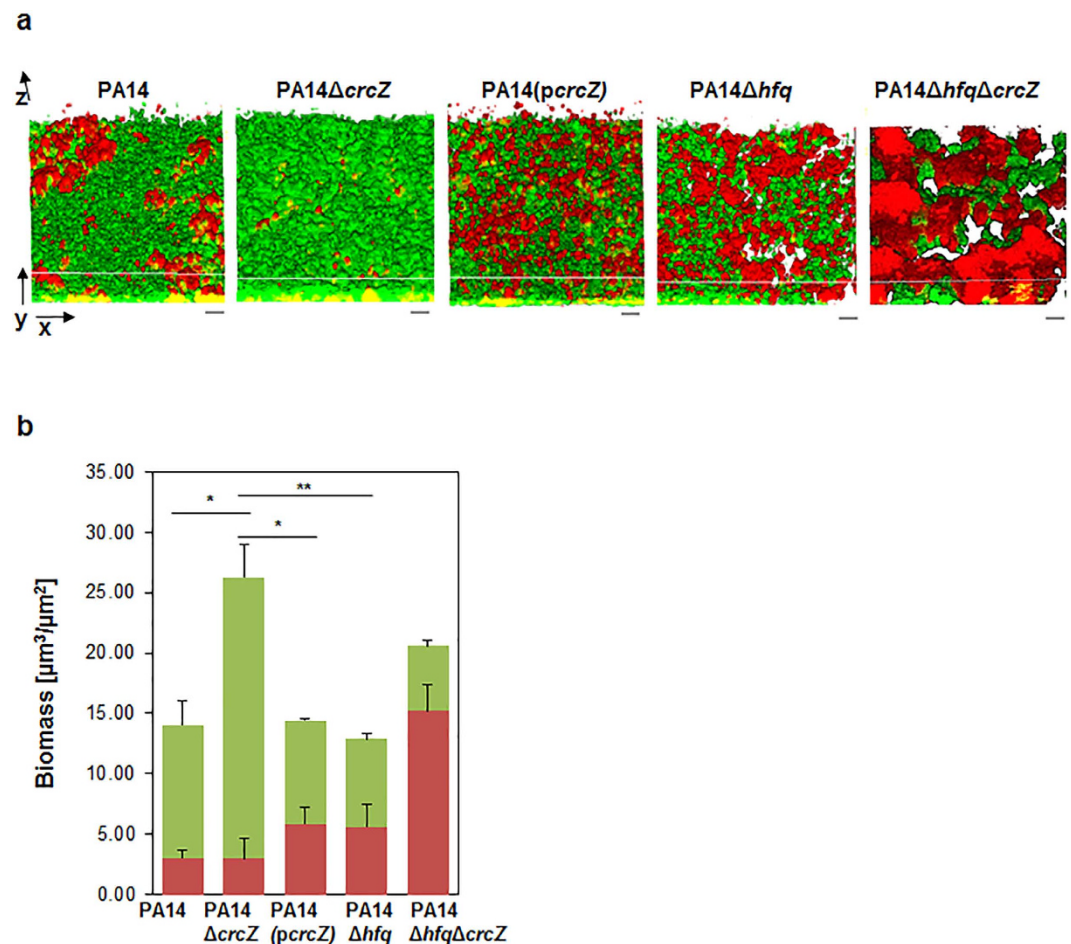


Figure 4. Biofilm formation of B-96 cultures of PA14, PA14Δ*crcZ*, PA14(*pcrcZ*), PA14Δ*hfq* and PA14Δ*hfq*Δ*crcZ*. (a) The distribution of live and death cells in B-96 cultures of PA14, PA14Δ*crcZ*, PA14(*pcrcZ*), PA14Δ*hfq* and PA14Δ*hfq*Δ*crcZ* were visualized by CLSM. Live cells (green) were stained with Syto 9 fluorescent dye, whereas dead cells (red) were visualized with propidium iodide. Three-dimensional images of biofilms (x, y, z-axes, scale bars represent 10 μm). The *CrcZ* levels in the different strains are shown in Supplementary Fig. S5a. (b) The biomass of live (green) and death (red) cells of B-96 cultures of PA14, PA14Δ*crcZ*, PA14(*pcrcZ*), PA14Δ*hfq* and PA14Δ*hfq*Δ*crcZ* were quantified from CLSM vertical image stacks using Comstat2 software⁵¹. Error bars, mean $\pm$ s.d. from three independent experiments. * $P < 0.05$, ** $P < 0.01$, analysed by one-way ANOVA with the Tukey's HSD post hoc test.

over-producing strain PA14(*pcrcZ*) and of PA14Δ*hfq* (Fig. 4). Moreover, the PA14Δ*hfq*Δ*crcZ* displayed a marked increase in dead cell biomass and decrease of viable cells (Fig. 4). Taken together, these results corroborate the idea the competition for Hfq by *CrcZ* perturbs metabolic pathways, which results in diminished anoxic growth and biofilm formation in *P. aeruginosa*.

The *CrcZ* levels are known to increase in the presence of poor carbon sources or at a low carbon to nitrogen ratio²⁰. Under these conditions, Hfq titration by *CrcZ* permits the uptake and utilization of non-preferred carbon sources¹⁵. When compared to planktonically grown cultures, the *CrcZ* levels were strongly increased in anoxic biofilms, which provides a further ambience where *CrcZ* can impact on Hfq function. Given the large Hfq regulon¹⁹ (Fig. 2) and the pivotal role of Hfq in PA physiology and virulence⁸, it appears worthwhile to explore *crcZ* regulation under a variety of conditions. A better understanding of the signals leading to *CrcZ* over-production could be valuable in view of developing strategies to attenuate the role of Hfq in PA pathogenicity.

CrcZ represents the first protein-binding RNA that limits biofilm formation. In contrast, several base-pairing sRNAs have been implicated in the regulation of biofilm formation in bacteria⁷. For example, the *Vibrio cholerae* VqmR RNA inhibits biofilm formation by translational silencing of the *vpsT* mRNA, encoding a transcriptional regulator of biofilm development³⁵, whereas the *Salmonella* Typhimurium ArcZ RNA positively regulates *rpoS*³⁶, which promotes biofilm formation³⁷. Interestingly, over-expression of ArcZ RNA changed the profile of Hfq-bound sRNAs and mRNAs, which suggested that ArcZ titrates Hfq and thereby might also post-transcriptionally cross-regulate other genes³⁶.

The biological function of *CrcZ* in limiting biofilm formation in PA might be associated with the complex environment in the CF lung. Recent studies indicated that polymorphonuclear leukocytes restrict the growth of PA in CF lungs, presumably by a high consumption of oxygen³⁸. This could be linked with an increased

CrcZ-mediated sequestration of Hfq under anoxic conditions and cessation of further biofilm formation, which in turn could be advantageous in terms of balancing the immune response and thus for establishing a long-term chronic infection.

Methods

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Supplementary Table S3. The synthetic cystic fibrosis sputum medium (SCFM) was prepared as described¹⁶. The concentration of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was increased to $100 \mu\text{M}$ and that of KNO_3 to 100 mM . This was done to allow for increased anaerobic biofilm formation after 96 h, which was required for the extraction of sufficient amounts of RNA for subsequent RNAseq analysis. For anoxic growth, 25 ml of bacterial cultures in SCFM were inoculated to an initial OD_{600} of 0.05 and then split in 1 ml aliquots into 5 ml polypropylene tubes. The cultures were incubated statically for 96 hours at 37°C in a 2.5-liter anaerobic jar containing a gas pack (AN25; AnaeroGen, Oxoid, United Kingdom).

RNAseq and CoIP RNAseq library construction and sequence analysis. The RNAseq analysis with total RNA prepared from two biological replicates of B-96 cultures was performed as recently described²². Hfq-bound RNAs were isolated from lysates of B-96 cultures after co-immunoprecipitation with Hfq-specific antibodies as described¹⁵. The Hfq-unbound fraction included all RNAs isolated from the supernatant after co-immunoprecipitation.

Total RNA from all samples was isolated 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. The Ribo-Zero rRNA Removal Kit (Gram-Negative Bacteria) Magnetic kit (Epicentre Biotechnologies) was used to deplete rRNA from samples used for RNAseq. Libraries were constructed using the NEBNext[®] Ultra[™] Directional RNA Library Prep Kit from Illumina. RNA sequencing has been performed at the Next Generation Sequencing Facility (VBCF, Vienna, Austria). The samples were subjected to Illumina 100 nt single end sequencing. PCR and index primers were removed from the raw reads with cutadapt³⁹ and quality control was performed with FastQC⁴⁰. Mapping of the samples against the PA UCBPP-PA14 reference genome (accession number NC_008463) was performed with segemehl^{41,42}. Mapped sequencing data were split by strand and further processed for automatic UCSC Genome Browser⁴³ visualization with the ViennaNGS toolbox⁴⁴. Read counting for subsequent DESeq⁴⁵ differential gene expression analysis was performed with BEDtools⁴⁶. Only reads corresponding to putative or confirmed regulatory RNAs of PA14 were considered in the analysis.

Northern-blot analyses. Total RNA was isolated by the hot phenol method as previously described¹⁰. The steady-state levels of CrcZ and 5S rRNA (loading control) were determined using $10 \mu\text{g}$ 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-labelled oligonucleotides: K3 (CrcZ) and I26 (5S rRNA), respectively (Supplementary Table S4). The hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

In vitro transcription of CrcZ. For *in vitro* transcription of CrcZ the AmpliScribe T7-Flash Transcription Kit (Epicentre Biotechnologies) was used according to the manufacturer's instructions. The 426 nt PCR fragment generated with the oligonucleotides E6 and C6 (Supplementary Table S4) served as template.

Microscale thermophoresis. Specific binding between CrcZ and Hfq was determined by microscale thermophoresis (MST)⁴⁷ using the Monolith NT.115 Green/Red apparatus (Nanotemper Technologies) at the Protein Technologies Facility (ProTech, VBCF, Vienna, Austria). Labelling of $20 \mu\text{M}$ of PA14 Hfq protein with NT-642 dye was performed using the Monolith[™] Antibody Labelling Kit RED-NHS (Amine Reactive; Nanotemper Technologies) according to the manufacturer's instructions. For the measurements, the concentration of the NT-642-labelled Hfq protein was kept constant (20 nM), whereas the concentrations of non-labelled *in vitro* transcribed CrcZ RNA varied from 1 to 1200 nM . The binding reactions were carried out in MST-buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM MgCl_2) supplemented with 0.1% Tween and 0.1% BSA. The reactants were initially incubated at 37°C for 30 min to enable CrcZ binding with Hfq. The samples were then loaded onto NT.115 hydrophilic capillaries (Nanotemper Technologies). The data for microscale thermophoresis analysis were recorded at 25°C using the red LED (excitation: 625 nm , emission: 680 nm); both, MST and LED Power at 40%. Data analyses were performed with NTAanalysis software.

NADH assay. The $[\text{NADH}]/[\text{NAD}^+]$ ratios were determined in B-96 cultures using an enzyme cycling-based colorimetric assay⁴⁸.

Microscopy. Image acquisition was performed at the MFPL BioOptics Facility with a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss, Germany) equipped with 405, 488, 555 and 639 nm laser diodes and two PMT channels for simultaneous monitoring of GFP (excitation, 488 nm ; emission, 517 nm) and propidium iodide (excitation, 543 nm ; emission, 565 nm). Images were obtained using a Plan-Apochromat 40x/1.3 Oil DIC objective. Simulated multichannel cross sections were generated using Fiji: an open-source platform for biological-image analysis and Volume Viewer Plugin⁴⁹. The quantitative analyses of acquired image stacks of biofilms was performed using Comstat2 software^{50,51}.

Statistical analysis. All experiments were performed at least in duplicate. The data shown were obtained with three biological replicates, unless indicated otherwise. Statistical significance was determined by one-way

ANOVA with the Tukey's honestly significant difference (HSD) post hoc test when more than two groups with normal distribution were compared. Levene's test was used to test for equality (homogeneity) of variances between tested groups. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant results.

Data availability. The raw sequencing data were deposited in the NCBI sequence read archive (SRA) as a study under the accession number SRP062593 (RNAseq) and SAMN05823591 (CoIP RNAseq), respectively. All other data generated or analysed during this study are included within this article and the Supplementary information or are available from the corresponding author upon request.

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Author Contributions

Conceived and designed the experiments: P.P., U.B. Performed the experiments: P.P., M.T. Analysed the data: P.P., M.T., M.W., E.S., S.H., U.B. Contributed reagents/materials/analysis tools: P.P., M.T., E.S., S.H., U.B. Wrote the paper: P.P., U.B.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

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5.1.1. Supplementary Figures and Tables

Supplementary information

Cross-regulation by CrcZ RNA controls anoxic biofilm formation in *Pseudomonas aeruginosa*

Petra Pusic¹, Muralidhar Tata¹, Michael T. Wolfinger^{2,3}, Elisabeth Sonnleitner¹, Susanne Häussler⁴ and Udo Bläsi^{1*}

¹Department of Microbiology, Immunobiology and Genetics, Center of Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter, Dr. Bohr-Gasse 9, 1030 Vienna, Austria, ²Institute of Theoretical Chemistry, University of Vienna, Währingerstrasse 17, 1090 Vienna, Austria, and ³Center for Anatomy and Cell Biology, Medical University of Vienna, Währingerstrasse 13, 1090 Vienna, Austria, ⁴Helmholtz Center for Infection Research, Molecular Bacteriology, Inhoffenstraße, 38124 Braunschweig, Germany

*Corresponding author.

22 **Supplementary text**

23 **Construction of the PA14 Δ *crcZ* mutant**

24 An in-frame deletion of the *crcZ* gene was constructed by homologous recombination⁵⁴.
25 Briefly, the 730-bp upstream- and 717-bp downstream region of the *crcZ* gene was PCR-
26 amplified using the primer pairs W100/Y100 and X100/Z100, respectively (Supplementary
27 Table S4). The PCR products were linked by an overlapping PCR to generate a fragment
28 containing the in-frame *crcZ* deletion. The fragment was cloned into the *KpnI* and *XbaI* sites
29 of plasmid pME3087 (Supplementary Table S3). Upon verification of the modification by
30 DNA sequencing, the resulting plasmid was mobilized into PA14 with the aid of the *E. coli*
31 strain HB101(pRK2013) and then chromosomally integrated through selection for
32 tetracycline resistance. Excision of the vector by a second crossover event was achieved by
33 enrichment for tetracycline-sensitive cells. The *crcZ* deletion was confirmed by standard
34 molecular methods.

35 **Construction of the PA14 Δ *hfq* Δ *crcZ* mutant**

36 A deletion of the *hfq* gene was introduced in PA14 Δ *crcZ* mutant as described above. Briefly,
37 the 717-bp upstream- and 799-bp downstream region of the *hfq* gene was PCR-amplified
38 using the primer pairs H71/J71 and K71/I71, respectively (Supplementary Table S4). The
39 PCR products were linked by an overlapping PCR to generate a fragment containing a 246-bp
40 deletion, which spans the entire *hfq* coding region except for the stop codon. The fragment
41 was cloned into the *BamHI* and *EcoRI* sites of plasmid pME3087 (Supplementary Table S3).
42 Upon verification of the modification by DNA sequencing, the resulting plasmid was
43 mobilized into PA14 Δ *crcZ* with the aid of the *E. coli* strain HB101(pRK2013) and then
44 chromosomally integrated through homologous recombination as described above. The *hfq*
45 deletion was confirmed by standard molecular methods.

RT-PCR

RNA was extracted from B-96 cells after co-immunoprecipitation with Hfq-specific antibodies from the co-immunoprecipitate (CoIP, Hfq-bound fraction) and from the remaining supernatant (SN, Hfq-unbound fraction). For cDNA synthesis, 500 ng RNA template was mixed with 250 ng of random primers (Promega). The mixture was then treated at 65°C for 5 min, followed by 5 min incubation on ice. The cDNA synthesis was performed with AMV reverse transcriptase (Promega) according to the manufacturer's instructions. 2 µl of cDNA derived from the RNA co-immunoprecipitated with Hfq and from the unbound total RNA, respectively, were used as a template for PCR amplification using the oligonucleotides C3 and E2 (Supplementary Table S4; CrcZ), T131 and U131 (Supplementary Table S4; ErsA) or D112 and E112 (Supplementary Table S4; RsmZ), respectively. The corresponding PCR products were 151 bp (CrcZ), 119 bp (ErsA) and 96 bp (RsmZ) in length. To confirm total DNA depletion of the RNA samples, the PCR reactions were performed with the same amount of RNA as used for the RT-PCR reaction without reverse transcriptase.

Western-blot analyses

Equal amounts of protein fractions were separated on 12.5% SDS-polyacrylamide gels and then electro-blotted to a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, followed by probing with rabbit anti-Hfq (Pineda). The antibody-antigen complexes were visualized with horseradish peroxidase conjugated secondary antibodies (Cell Signaling Technology) using the enhanced chemiluminescent (ECL) HRP substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) and ChemiDoc Touch Imaging System (BioRad).

Metabolic activity assay using the *rrnBP1*-GFP_{AGA} bioreporter

The delivery plasmid for miniTn7(Gm)-P_{*rrnBP1*}-*gfp*-AGA-a²⁴ and the helper plasmid pUX-BF13 (encodes the transposase genes) were introduced together by electroporation in strains PA14, PA14Δ*hfq* and PA14Δ*crcZ*, respectively, as previously described⁵⁵. To monitor the metabolic state of the cells in anoxic biofilms, Gfp (excitation, 488 nm; emission, 517 nm) synthesis was measured in 24h-intervals for 4 days.

Biofilm assays

For the static crystal violet assay³⁴ the cultures were inoculated as described in the Methods section and incubated for 96h. Then, the contents of tubes were removed, washed 3 times with water and air dried. 1.2 ml of 0.1% (w/v) crystal violet was added followed by incubation at room temperature for 10 min. The tubes were washed and air dried. The stain attached to the tubes was solubilized using 95% ethanol. Biofilm formation was assessed by measuring the optical density of each sample at a wavelength of 595 nm.

83 **Supplementary References**

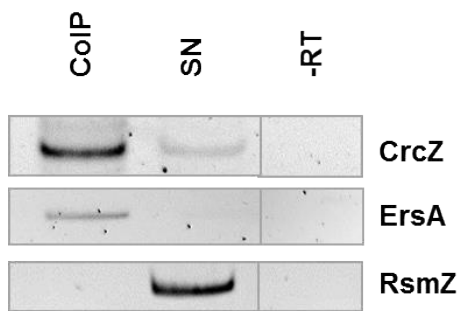
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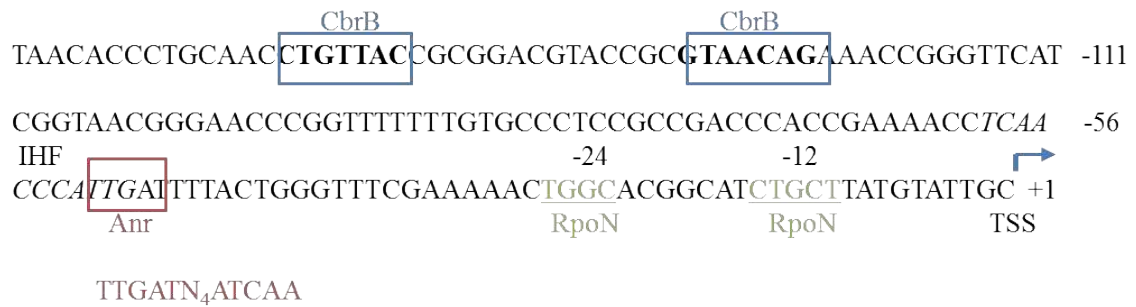
Supplementary Figures



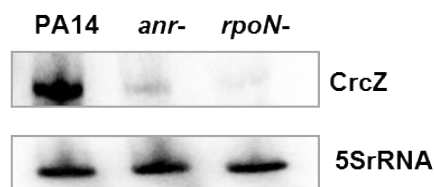
Supplementary Figure S1. Hfq co-immunoprecipitates with CrcZ and ErsA in B-96 cultures.

RNA was extracted from lysates of B-96 cultures after co-immunoprecipitation with Hfq-specific antibodies (CoIP; Hfq-bound fraction) and from the remaining supernatant (SN, Hfq-unbound fraction). Equal amounts were used for RT-PCR with specific primers for CrcZ, ErsA and RsmZ as described in Supplementary text. -RT, mock experiment with RNA obtained from the Hfq-bound fraction (CoIP) in the absence of reverse transcriptase.

a



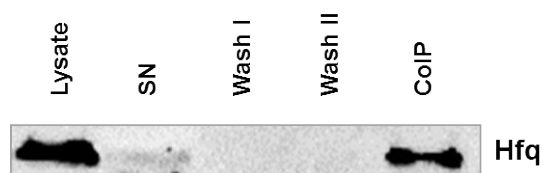
b



Supplementary Figure S2. Anr stimulates *crcZ* expression in B-96 cultures.

a Sequence of the *crcZ* promoter region. The transcriptional start site (TSS, +1) is marked with an arrow. The RpoN promoter sequence is shown in green and underlined. A putative IHF binding site is shown in italics. The CbrB binding sites and a partial putative recognition motif for Anr are shown by blue and red boxes, respectively²¹. The Anr consensus motif⁵⁶ is shown below.

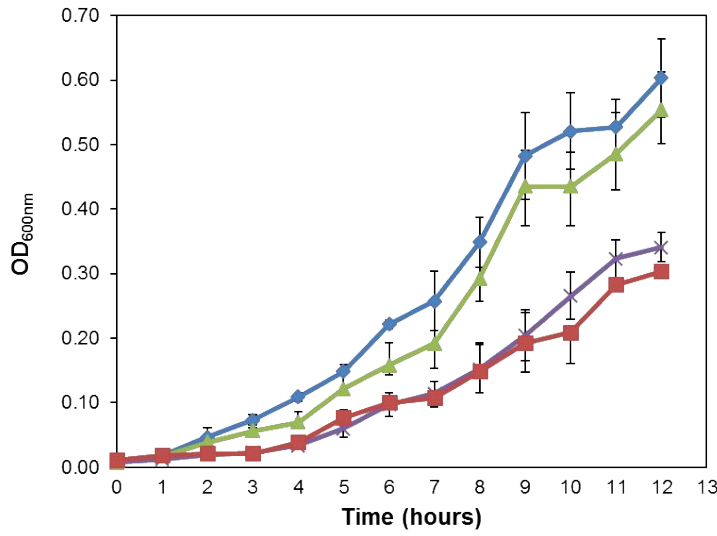
b Total RNA was isolated from B-96 cultures of PA14, PA14*anr*⁻ and PA14*rpoN*⁻, and 10 µg of total RNA was used for the Northern-blot analysis. 5S rRNA served as a loading control.



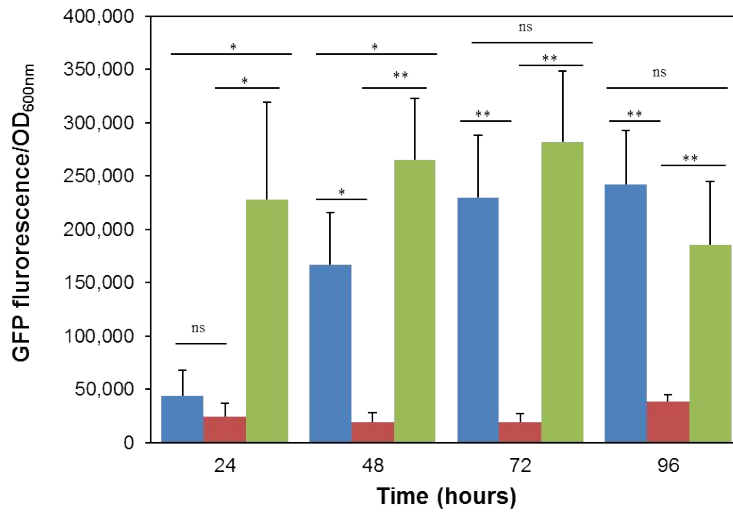
Supplementary Figure S3. Presence of Hfq in different steps during co-immunoprecipitation.

Samples were withdrawn from lysates of B-96 cultures during different steps of co-immunoprecipitation with Hfq-specific antibodies and examined by western-blotting for the presence of Hfq as described in Supplementary text. Lysate: initial lysate (input); SN (supernatant, Hfq-unbound fraction); wash I and II represent washing fractions; CoIP (co-immunoprecipitate, Hfq bound fraction).

a



b



139

140 **Supplementary Figure S4. Hfq is pivotal for anoxic growth and metabolism.**

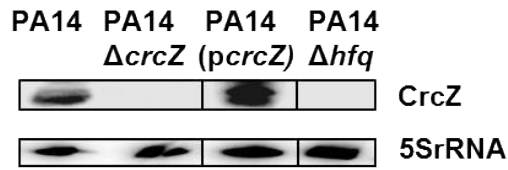
141 a The strains PA14 (blue bar), PA14ΔcrcZ (green bar), PA14(pcrcZ) (violet bar) and
 142 PA14Δhfq (red bar), were grown anaerobically for 12 hours in SCFM. Cell growth was
 143 monitored by measuring the optical density at 600 nm (OD_{600nm}).

144 b Metabolic activity of strains PA14 (blue bar), PA14Δhfq (red bar) and PA14ΔcrcZ (green
 145 bar) monitored with the *PrnBP1-gfp*(AGA) bioreporter after growth in SCF-medium. The
 146 synthesis of Gfp (excitation, 488 nm; emission, 517 nm), as well as the OD₆₀₀ was measured
 147 every 24 h for 4 days. Error bars, mean ± s.d. from a representative of three independent

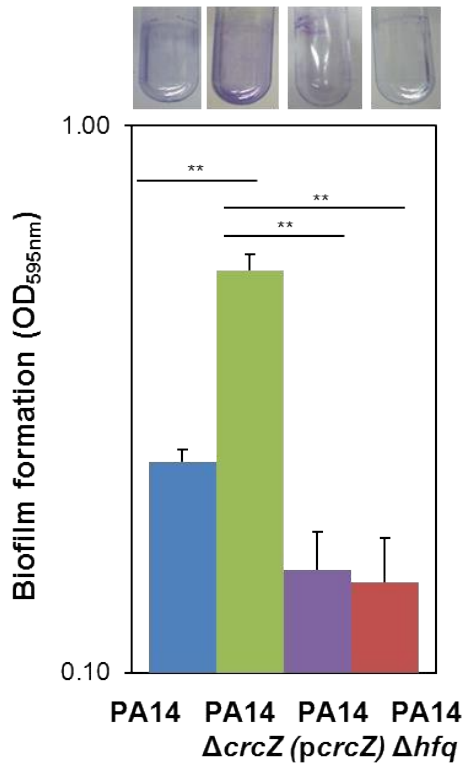
148 experiments. N = 3 biological replicates. ns (non-significant), * $P < 0.05$, ** $P < 0.01$,
149 analysed by one-way ANOVA with the Tukey's HSD post hoc test.

150

a



b



Supplementary Figure S5. Biofilm formation of B-96 cultures of PA14, PA14Δ*crcZ*, PA14(*pcrcZ*) and PA14Δ*hfq*.

a Steady-state levels of CrcZ in B-96 cultures of PA14, PA14Δ*crcZ*, PA14(*pcrcZ*) and PA14Δ*hfq* (see also Fig. 4a). 10 μg of total RNA was used for the Northern-blot analysis with the CrcZ specific probe. 5SrRNA served as a loading control.

b Top, biofilm formation was assessed by a crystal violet assay. Bottom, graphical representation of the results obtained with B-96 cultures of PA14 (blue bar), PA14Δ*crcZ* (green bar), PA14(*pcrcZ*) (violet bar) and PA14Δ*hfq* (red bar). Error bars, mean ± s.d. from a

160 representative of three independent experiments. N = 3 biological replicates with 3 technical
161 replicates. ** $P < 0.01$, analysed by one-way ANOVA with the Tukey's HSD post hoc test.

162

Supplementary Table S1. Known and putative regulatory RNAs found in the Hfq-bound and unbound fraction. All RNAs with less than 150 reads were excluded.

sRNA_id ^a	Start ^b	End ^c	Strand ^d	Hfq bound ^e fraction	Hfq unbound ^e fraction
CrcZ_igr2625 (SPA0092)	5580518	5580924	+	2028048	29087
SPA0072 (tmRNA_ssrA)	4749699	4750100	+	368491	93138
SPA0094 (6SRNA_ssrS_igr2888)	6156499	6156800	+	45301	51387
SPA0091 (rnpB)	5119799	5120100	-	39037	25631
P30	5580674	5580896	-	24961	789
SPA0085 (RsmZ)	1500299	1500500	+	5413	70581
SPA0069 (RsmY)	596799	597000	+	4968	17203
SPA0011	2677699	2678200	+	2271	2425
SPA0012	3515399	3515700	+	2002	367
SPA0142	139699	140100	+	1582	64
SPA0104	1651699	1651900	+	1572	269
SPA0105	1648399	1649100	+	1475	51
SPA0021	5288099	5288500	+	848	257
rgsA_igr1076 (P16)	2250484	2250742	-	744	209
SPA0122 (ErsA)	6456399	6456600	-	734	89
SPA0076f (4.5S_igr1863)	3975599	3975800	-	637	1024
large_SRP	3975616	3975841	-	635	1024
SPA0124	4105199	4105600	-	623	657
PA14sr_035 (igr576)	1198079	1199053	-	608	171
PA14sr_090	3601259	3601463	-	560	42
igr1766	3758216	3764041	+	559	1
SPA0178	894999	895300	-	533	45
igr1944	4155629	4160668	+	522	1
SPA0145	1774199	1774400	-	518	24
SPA0013	3974899	3975200	-	461	20
SPA0025	2935699	2937400	-	358	2147
igr1276	2604153	2604747	-	346	51
SPA0147	2236399	2237000	-	279	63
SPA0126	1836399	1836900	-	250	3
igr329	642266	643346	-	231	13
SPA0073 (sRNA 645)	4444699	4445000	+	202	20
SPA0125	2464699	2465200	+	200	22
SPA0119	5951199	5952700	-	197	1
SPA0166	4085299	4085600	+	184	6
SPA0117	2154399	2154700	-	178	8
PA14sr_078	2924519	2925530	-	173	1
PA14sr_065	2465066	2465518	+	172	36
SPA0121	6444599	6445100	-	169	36

^{a-d} Annotation based on location information according to¹⁷. ^e Sequencing reads averaged from two independent experiments.

Supplementary Table S2. Differential abundance of transcripts in B-96 cultures of PA14 and PA14Δhfq.

a Transcripts with increased abundance in B-96 cultures of PA14Δhfq when compared with PA14. A fold change cut-off of 4 and a p-value above 0.05 were applied. The colour coding of different functional classes corresponds to those shown in Fig. 2a.

PA14_id ^b	Gene ^c	Description ^d	Fold Change	log2Fold Change	pval	padj
Adaptation, Protection						
PA14_33690	<i>pvdE</i>	pyoverdine biosynthesis protein PvdE	4.05	2.02	1.83E-33	4.44E-32
PA14_49410		cold-shock protein	5.50	2.46	3.90E-58	2.37E-56
PA14_58000	<i>sodM</i>	superoxide dismutase	6.87	2.78	1.23E-20	1.54E-19
Amino acid biosynthesis and metabolism						
PA14_05750	<i>gltX</i>	oxidoreductase	17.34	4.12	9.97E-49	4.38E-47
PA14_37560	<i>asnB</i>	asparagine synthetase, glutamine-hydrolysing	7.84	2.97	1.77E-81	1.93E-79
PA14_69990	<i>dadX</i>	alanine racemase	7.04	2.82	8.54E-41	2.86E-39
PA14_71180		acetylornithine deacetylase	4.21	2.07	8.97E-12	6.13E-11
PA14_73090		hypothetical protein	6.56	2.71	1.71E-39	5.35E-38
Glycine and serine degradation						
PA14_32985	<i>gcvH2</i>	glycine cleavage system protein H	10.22	3.35	3.36E-62	2.25E-60
PA14_33000	<i>gcvP2</i>	glycine dehydrogenase	6.92	2.79	5.04E-75	4.72E-73
PA14_33010	<i>glyA2</i>	serine hydroxymethyltransferase	7.43	2.89	1.28E-72	1.11E-70
PA14_33030	<i>sdaA</i>	L-serine dehydratase	4.50	2.17	1.33E-41	4.55E-40
Branched-chain amino acid degradation						
PA14_18120	<i>mmsA</i>	methylmalonate-semialdehyde dehydrogenase	42.80	5.42	1.44E-227	1.69E-224
PA14_18140	<i>mmsB</i>	3-hydroxyisobutyrate dehydrogenase	74.19	6.21	5.91E-203	4.35E-200
PA14_35490	<i>lpdV</i>	dihydrolipoamide dehydrogenase	19.24	4.27	1.35E-142	3.32E-140
PA14_35500	<i>bkdB</i>	branched-chain alpha-keto acid dehydrogenase subunit E2	31.43	4.97	5.92E-152	1.66E-149
PA14_35520	<i>bkdA2</i>	2-oxoisovalerate dehydrogenase subunit beta	12.91	3.69	1.22E-49	5.47E-48
PA14_35530	<i>bkdA1</i>	2-oxoisovalerate dehydrogenase subunit alpha	7.36	2.88	9.55E-44	3.57E-42
PA14_19920		branched-chain alpha-keto acid dehydrogenase subunit E2	5.93	2.57	6.34E-35	1.63E-33
Biosynthesis of cofactors, prosthetic groups and carriers						
PA14_02420	<i>atsK</i>	hypothetical protein	55.33	5.79	9.83E-176	5.27E-173
PA14_13850	<i>moaA</i>	molybdenum cofactor	3.88	1.95	2.16E-09	1.17E-08

		biosynthesis protein A				
PA14_20140	<i>fpr</i>	ferredoxin--NADP+ reductase	7.80	2.96	3.12E-84	3.75E-82
PA14_31510		short-chain dehydrogenase	5.57	2.48	1.21E-20	1.53E-19
PA14_61750	<i>ipk</i>	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	5.51	2.46	2.08E-60	1.32E-58
PA14_66550	<i>hemE</i>	uroporphyrinogen decarboxylase	4.83	2.27	7.76E-38	2.25E-36
PQQ biosynthesis						
PA14_38780	<i>pqqE</i>	pyrroloquinoline quinone biosynthesis protein PqqE	6.55	2.71	2.45E-65	1.72E-63
PA14_38790	<i>pqqD</i>	pyrroloquinoline quinone biosynthesis protein PqqD	5.93	2.57	1.97E-46	8.06E-45
PA14_38800	<i>pqqC</i>	pyrroloquinoline quinone biosynthesis protein PqqC	5.73	2.52	1.07E-19	1.28E-18
PA14_38820	<i>pqqB</i>	pyrroloquinoline quinone biosynthesis protein PqqB	7.75	2.95	2.58E-71	2.14E-69
PA14_38825	<i>pqqA</i>	coenzyme PQQ synthesis protein PqqA	10.33	3.37	1.15E-13	9.14E-13
Carbon compound catabolism						
PA14_02760	<i>catI</i>	CoA transferase, subunit A	4.04	2.01	9.05E-10	5.13E-09
PA14_20560	<i>amiE</i>	acylamide amidohydrolase	6.29	2.65	7.06E-56	3.96E-54
PA14_32160	<i>antA</i>	anthranilate dioxygenase large subunit	8.73	3.13	1.83E-25	2.97E-24
PA14_37550		ring-hydroxylating dioxygenase small subunit	8.30	3.05	7.68E-44	2.90E-42
PA14_37570		ring-hydroxylating dioxygenase, large terminal subunit	14.33	3.84	1.79E-127	3.90E-125
PA14_38590	<i>bdhA</i>	3-hydroxybutyrate dehydrogenase	6.38	2.67	1.60E-64	1.11E-62
PA14_70640	<i>rubA1</i>	rubredoxin 1	6.91	2.79	3.50E-53	1.82E-51
Malonate metabolism						
PA14_02580	<i>mdcD</i>	malonate decarboxylase subunit beta	4.55	2.19	9.33E-17	9.36E-16
PA14_02590	<i>mdcE</i>	malonate decarboxylase subunit gamma	8.21	3.04	2.05E-52	1.01E-50
Central intermediary metabolism						
PA14_09290	<i>pchG</i>	pyochelin biosynthetic protein PchG	3.89	1.96	4.08E-11	2.61E-10
PA14_36330	<i>hcnA</i>	hydrogen cyanide synthase HcnA	4.30	2.10	9.90E-33	2.31E-31
PA14_40040		hypothetical protein	60.18	5.91	8.52E-197	5.57E-194
PA14_46960	<i>ggt</i>	gamma-glutamyltranspeptidase	4.13	2.05	7.56E-24	1.13E-22
Sulfur metabolism						
PA14_02310	<i>atsA</i>	arylsulfatase	23.06	4.53	1.76E-154	5.45E-152
PA14_40770	<i>cysI</i>	sulfite reductase	4.43	2.15	2.60E-46	1.06E-44
Chaperones & heat shock proteins						
PA14_20570	<i>amiB</i>	chaperone	7.39	2.88	8.11E-36	2.18E-34

Chemotaxis						
PA14_55960	<i>pctC</i>	chemotactic transducer PctC	5.93	2.57	3.73E-08	1.78E-07
Che operon						
PA14_02180	<i>cheB</i>	chemotaxis-specific methyltransferase	8.09	3.02	6.74E-32	1.52E-30
PA14_02190	<i>cheD</i>	hypothetical protein	16.43	4.04	3.07E-57	1.85E-55
PA14_02200	<i>cheR</i>	chemotaxis protein methyltransferase	16.67	4.06	5.26E-83	6.08E-81
PA14_02220		chemotaxis transducer	13.31	3.73	3.76E-114	6.71E-112
PA14_02230	<i>cheW</i>	purine-binding chemotaxis protein	12.38	3.63	9.85E-73	8.66E-71
DNA replication, recombination, modification and repair						
PA14_70600	<i>hupA</i>	HU family DNA-binding protein	3.92	1.97	1.51E-07	6.84E-07
Energy metabolism						
PA14_65080	<i>ygiR</i>	hypothetical protein	3.95	1.98	2.26E-37	6.37E-36
PA14_65810		hypothetical protein	6.22	2.64	5.39E-23	7.61E-22
PA14_02450		NAD(P) transhydrogenase subunit alpha part 1	4.69	2.23	8.67E-30	1.80E-28
PA14_02470	<i>pntB</i>	pyridine nucleotide transhydrogenase subunit beta	4.95	2.31	2.78E-29	5.57E-28
PA14_02680	<i>aldH</i>	aldehyde dehydrogenase	15.71	3.97	4.76E-17	4.86E-16
PA14_38750		iron-containing alcohol dehydrogenase	5.68	2.51	7.86E-60	4.87E-58
Pyruvate metabolism						
PA14_19910	<i>pdhB</i>	pyruvate dehydrogenase E1 component, beta chain	4.23	2.08	6.05E-13	4.54E-12
PA14_71720	<i>oadA</i>	pyruvate carboxylase subunit B	6.54	2.71	8.97E-67	6.77E-65
PA14_71740	<i>accC</i>	acetyl-CoA carboxylase biotin carboxylase subunit	8.12	3.02	2.75E-53	1.45E-51
Cytochrome c oxidases						
PA14_01300	<i>coxA</i>	cytochrome c oxidase subunit I	6.51	2.70	1.22E-46	5.11E-45
PA14_01310	<i>coxG</i>	cytochrome C oxidase assembly protein	10.69	3.42	5.89E-38	1.72E-36
PA14_01320	<i>coIII</i>	cytochrome c oxidase subunit III	8.67	3.12	8.97E-54	4.81E-52
PA14_40510	<i>ccoN-2</i>	cbb3-type cytochrome c oxidase subunit I	9.35	3.23	2.62E-37	7.36E-36
PA14_44340	<i>ccoN</i>	cbb3-type cytochrome c oxidase subunit I	8.50	3.09	1.47E-82	1.67E-80
PA14_44350	<i>ccoO</i>	cbb3-type cytochrome c oxidase subunit II	13.84	3.79	2.69E-102	4.06E-100
PA14_44360	<i>ccoP</i>	cytochrome c oxidase, cbb3-type subunit III	10.57	3.40	4.34E-89	5.56E-87
PA14_44370	<i>ccoN</i>	cbb3-type cytochrome c oxidase subunit I	5.27	2.40	2.07E-37	5.89E-36
PA14_45330	<i>ccmE</i>	cytochrome c-type biogenesis protein CcmE	3.97	1.99	1.71E-05	6.02E-05
PA14_57560		cytochrome b	4.06	2.02	7.85E-41	2.64E-39
PA14_60700	<i>ccpR</i>	cytochrome c551 peroxidase	6.39	2.68	2.51E-54	1.37E-52

Ethanol oxidation

PA14_38840	<i>exaC</i>	NAD ⁺ dependent acetaldehyde dehydrogenase	127.05	6.99	7.40E- 255	1.45E- 251
PA14_38850	<i>exaB</i>	cytochrome c550	169.04	7.40	6.78E- 160	2.35E- 157
PA14_38860	<i>exaA</i>	quinoprotein alcohol dehydrogenase	260.81	8.03	0.00E+00	0.00E+00

Denitrification (*nor* operon; nitric-oxide reductase)

PA14_06810	<i>norC</i>	nitric-oxide reductase subunit C	3.89	1.96	5.89E-19	6.76E-18
PA14_06830	<i>norB</i>	nitric-oxide reductase subunit B	4.47	2.16	8.15E-38	2.35E-36
PA14_06840	<i>norD</i>	dinitrification protein NorD	4.64	2.21	9.57E-11	5.95E-10
PA14_11690	<i>ppa</i>	inorganic pyrophosphatase	3.98	1.99	2.21E-13	1.72E-12

Denitrification (*nar* operon; membrane-bound nitrate reductase)

PA14_13780	<i>narG</i>	respiratory nitrate reductase alpha subun	4.53	2.18	4.04E-45	1.61E-43
PA14_13800	<i>narH</i>	respiratory nitrate reductase beta subuni	5.35	2.42	4.42E-53	2.28E-51
PA14_13810	<i>narJ</i>	respiratory nitrate reductase delta chain	4.47	2.16	1.32E-30	2.85E-29
PA14_13830	<i>narI</i>	respiratory nitrate reductase gamma chain	5.20	2.38	2.43E-38	7.25E-37

Denitrification (*nap* operon; periplasmic nitrate reductase)

PA14_49210	<i>napE</i>	periplasmic nitrate reductase NapE	4.34	2.12	1.13E-27	2.03E-26
PA14_49230	<i>napD</i>	NapD protein of periplasmic nitrate reductase	4.03	2.01	1.46E-09	8.07E-09
PA14_49250	<i>napA</i>	nitrate reductase catalytic subunit	7.81	2.96	2.87E-69	2.32E-67
PA14_49260	<i>napB</i>	cytochrome c-type protein NapB precursor	11.05	3.47	1.72E-40	5.66E-39
PA14_49270	<i>napc</i>	cytochrome c-type protein NapC	11.71	3.55	1.41E-56	8.31E-55

TCA cycle

PA14_58030	<i>fumC</i>	fumarate hydratase	4.17	2.06	1.12E-42	3.95E-41
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ATP-synthase

PA14_73310	<i>atpB</i>	F0F1 ATP synthase subunit A	4.03	2.01	4.45E-40	1.43E-38
PA14_73320	<i>atpI</i>	F0F1 ATP synthase subunit I	4.85	2.28	2.88E-48	1.23E-46

Fatty acid and phospholipid metabolism

PA14_18150	<i>acsL</i>	acetyl-coa synthetase	15.93	3.99	9.47E- 136	2.23E- 133
PA14_31530		acyl-CoA thiolase	4.96	2.31	6.03E-07	2.55E-06
PA14_39990		hypothetical protein	113.00	6.82	1.01E- 252	1.49E- 249
PA14_65820		acyl-CoA dehydrogenase	7.40	2.89	1.07E-11	7.24E-11
PA14_69660	<i>lppL</i>	lipopeptide LppL	4.34	2.12	4.33E-05	1.46E-04

Hypothetical, unclassified, unknown

PA14_01340		hypothetical protein	9.41	3.23	1.41E-35	3.71E-34
PA14_04020		hypothetical protein	5.99	2.58	1.99E-40	6.48E-39
PA14_05110		hypothetical protein	5.30	2.41	1.56E-12	1.14E-11
PA14_07355		hypothetical protein	37.61	5.23	1.88E- 186	1.11E- 183
PA14_07370		hypothetical protein	5.44	2.44	1.31E-29	2.64E-28

PA14_07650	<i>ycgB</i>	SpoVR family protein	6.12	2.61	2.13E-32	4.88E-31
PA14_07660		hypothetical protein	4.55	2.18	6.62E-43	2.38E-41
PA14_13920		hypothetical protein	4.14	2.05	5.32E-15	4.68E-14
PA14_14540		hypothetical protein	3.98	1.99	2.28E-23	3.33E-22
PA14_20840		hypothetical protein	4.00	2.00	5.10E-25	8.09E-24
PA14_28390		hypothetical protein	10.41	3.38	9.75E-42	3.38E-40
PA14_29470		hypothetical protein	6.22	2.64	9.62E-43	3.41E-41
PA14_34050		hypothetical protein	4.89	2.29	1.49E-18	1.66E-17
PA14_34070		hypothetical protein	3.90	1.96	9.37E-24	1.39E-22
PA14_34080		hypothetical protein	4.08	2.03	7.09E-10	4.06E-09
PA14_34100		hypothetical protein	4.19	2.07	1.42E-09	7.86E-09
PA14_34130		hypothetical protein	5.29	2.40	1.36E-46	5.65E-45
PA14_37410		hypothetical protein	3.87	1.95	2.35E-25	3.80E-24
PA14_37520		hypothetical protein	4.83	2.27	2.27E-28	4.27E-27
PA14_38880		hypothetical protein	505.05	8.98	6.91E-175	3.13E-172
PA14_38920		hypothetical protein	29.89	4.90	5.09E-124	1.07E-121
PA14_38990		hypothetical protein	32.29	5.01	1.70E-170	6.69E-168
PA14_39000		hypothetical protein	58.99	5.88	6.05E-20	7.36E-19
PA14_40010		hypothetical protein	155.00	7.28	8.30E-271	2.44E-267
PA14_40020		hypothetical protein	46.22	5.53	2.12E-175	1.04E-172
PA14_40050		hypothetical protein	38.89	5.28	8.11E-112	1.40E-109
PA14_40100		hypothetical protein	4.94	2.30	6.08E-30	1.28E-28
PA14_40110		hypothetical protein	4.94	2.30	1.08E-09	6.04E-09
PA14_40520		hypothetical protein	4.54	2.18	4.70E-16	4.46E-15
PA14_40560		hypothetical protein	7.21	2.85	8.85E-69	6.86E-67
PA14_40750		hypothetical protein	4.62	2.21	5.04E-22	6.75E-21
PA14_40780		hypothetical protein	4.27	2.09	8.71E-40	2.76E-38
PA14_41760		hypothetical protein	4.08	2.03	3.03E-19	3.54E-18
PA14_41790		hypothetical protein	5.53	2.47	3.34E-37	9.31E-36
PA14_42410		hypothetical protein	8.04	3.01	7.81E-52	3.77E-50
PA14_44430		hypothetical protein	4.65	2.22	9.04E-43	3.23E-41
PA14_46380		hypothetical protein	4.07	2.03	1.22E-16	1.21E-15
PA14_46390		hypothetical protein	5.20	2.38	5.00E-23	7.12E-22
PA14_47410	<i>ycgN</i>	hypothetical protein	4.94	2.31	4.32E-21	5.53E-20
PA14_49850		hypothetical protein	4.11	2.04	6.53E-12	4.56E-11
PA14_51940		hypothetical protein	5.06	2.34	3.20E-21	4.14E-20
PA14_51950		hypothetical protein	4.44	2.15	6.76E-35	1.73E-33
PA14_52910		hypothetical protein	10.54	3.40	2.00E-14	1.69E-13
PA14_54870		hypothetical protein	3.91	1.97	7.75E-39	2.37E-37
PA14_55760		hypothetical protein	3.89	1.96	5.51E-08	2.59E-07
PA14_58010		hypothetical protein	5.29	2.40	7.86E-50	3.56E-48
PA14_60480		hypothetical protein	8.62	3.11	1.49E-72	1.28E-70
PA14_68620		hypothetical protein	4.44	2.15	8.03E-33	1.89E-31
PA14_71190		hypothetical protein	38.36	5.26	5.70E-21	7.25E-20

Membrane proteins						
PA14_02060		hypothetical protein	5.32	2.41	5.75E-04	1.61E-03
PA14_22350	<i>actP</i>	acetate permease	5.30	2.41	1.18E-44	4.57E-43
PA14_28400	<i>oprQ</i>	outer membrane OprD family porin	12.21	3.61	1.40E-121	2.65E-119
PA14_29660		hypothetical protein	4.22	2.08	3.02E-08	1.46E-07
PA14_32720		transmembrane sensor	5.21	2.38	6.05E-29	1.20E-27
PA14_38950		hypothetical protein	27.61	4.79	9.52E-133	2.16E-130
PA14_39810		transmembrane sensor	5.21	2.38	6.15E-36	1.68E-34
PA14_39830		hypothetical protein	4.34	2.12	2.20E-15	1.99E-14
PA14_40540		hypothetical protein	5.68	2.51	2.39E-33	5.78E-32
PA14_46650		transmembrane sensor	4.63	2.21	1.85E-29	3.73E-28
PA14_64690		transmembrane sensor	4.83	2.27	4.54E-38	1.34E-36
Motility & Attachment						
Type IV pili						
PA14_05190	<i>pilU</i>	twitching motility protein PilU	4.28	2.10	4.58E-11	2.92E-10
PA14_05340	<i>pilI</i>	twitching motility protein PilI	4.18	2.06	1.68E-27	2.98E-26
PA14_05380	<i>pilK</i>	methyltransferase PilK	3.92	1.97	8.85E-29	1.71E-27
PA14_58730	<i>pilA</i>	type IV pilin structural subunit	3.93	1.97	3.10E-39	9.60E-38
PA14_66630	<i>pilP</i>	type 4 fimbrial biogenesis protein PilP	5.09	2.35	1.05E-22	1.45E-21
Nucleotide biosynthesis and metabolism						
PA14_05740	<i>pydA</i>	dihydropyrimidine dehydrogenase	31.43	4.97	1.42E-76	1.39E-74
PA14_05770	<i>dhT</i>	phenylhydantoinase	6.50	2.70	2.84E-61	1.86E-59
PA14_05790		transporter	6.49	2.70	4.35E-15	3.84E-14
PA14_05810	<i>amaB</i>	allantoate amidohydrolase	5.47	2.45	1.21E-31	2.71E-30
PA14_44740	<i>xdhB</i>	xanthine dehydrogenase	6.73	2.75	7.95E-51	3.74E-49
PA14_44760		xanthine dehydrogenase accessory factor X	4.49	2.17	1.20E-24	1.86E-23
PA14_53290	<i>trxB2</i>	thioredoxin reductase 2	3.87	1.95	5.64E-10	3.27E-09
PA14_61460	<i>hpt</i>	hypoxanthine-guanine phosphoribosyltransferase	3.97	1.99	1.12E-15	1.03E-14
PA14_61770	<i>prs</i>	ribose-phosphate pyrophosphokinase	3.87	1.95	1.16E-37	3.31E-36
Protein secretion/export apparatus						
PA14_19680		hypothetical protein	9.87	3.30	6.99E-10	4.01E-09
PA14_19690		hypothetical protein	4.97	2.31	8.83E-04	2.40E-03
PA14_40240		ABC transporter ATP-binding protein/permease	4.59	2.20	2.02E-22	2.74E-21
PA14_55790		hypothetical protein	16.50	4.04	4.69E-106	7.26E-104
PA14_55800		hypothetical protein	3.86	1.95	2.25E-21	2.93E-20
PA14_55860		hypothetical protein	7.14	2.84	3.00E-05	1.03E-04
PA14_55880		hypothetical protein	14.68	3.88	1.11E-52	5.53E-51
Type III secretion system						
PA14_42350	<i>pscC</i>	Type III secretion outer membrane protein PscC precursor	4.09	2.03	4.69E-24	7.14E-23

PA14_42360	<i>pscB</i>	type III export apparatus protein	7.15	2.84	2.71E-26	4.56E-25
PA14_42380	<i>exsD</i>	hypothetical protein	4.20	2.07	7.78E-14	6.30E-13
PA14_42400	<i>exsB</i>	exoenzyme S synthesis protein B	5.53	2.47	1.16E-13	9.17E-13
PA14_42440	<i>popD</i>	translocator outer membrane protein PopD precursor	7.20	2.85	7.40E-36	2.01E-34
PA14_42450	<i>popB</i>	translocator protein PopB	7.31	2.87	2.12E-39	6.60E-38
PA14_42470	<i>pcrV</i>	type III secretion protein PcrV	11.31	3.50	2.19E-37	6.20E-36
PA14_42570	<i>pscN</i>	type III secretion system ATPase	21.64	4.44	1.56E-48	6.75E-47
Type II secretion system						
PA14_55890	<i>cpaF2</i>	type II secretion system protein	10.70	3.42	3.17E-43	1.17E-41
PA14_55900		hypothetical protein	8.51	3.09	2.31E-26	3.92E-25
PA14_55920		type II secretion system protein	7.98	3.00	3.96E-50	1.81E-48
PA14_55930		pilus assembly protein	5.32	2.41	7.30E-17	7.35E-16
PA14_55940		hypothetical protein	42.06	5.39	3.05E-221	2.57E-218
Putative enzymes						
PA14_02435		hypothetical protein	30.64	4.94	2.29E-110	3.85E-108
PA14_11810		aldehyde dehydrogenase	21.05	4.40	3.98E-84	4.69E-82
PA14_30500		acyl-CoA dehydrogenase	4.64	2.21	1.05E-29	2.15E-28
PA14_37530		hydrolase	6.27	2.65	6.98E-51	3.31E-49
PA14_40030		hypothetical protein	100.91	6.66	2.60E-226	2.55E-223
PA14_51050	<i>pcd</i>	aldehyde dehydrogenase	5.75	2.52	1.79E-32	4.14E-31
PA14_52900		acyl-CoA dehydrogenase	9.81	3.29	5.24E-36	1.44E-34
PA14_54630		acyl-CoA dehydrogenase	8.86	3.15	9.31E-66	6.69E-64
PA14_54640		enoyl-CoA hydratase	6.88	2.78	3.91E-52	1.90E-50
PA14_54660		enoyl-CoA hydratase/isomerase	7.87	2.98	9.03E-57	5.37E-55
PA14_56840		hypothetical protein	4.80	2.26	1.04E-40	3.46E-39
Secreted Factors (toxins, enzymes, alginate)						
PA14_26020		aminopeptidase	4.24	2.08	3.87E-19	4.49E-18
Phenazine biosynthesis						
PA14_09470	<i>phzB1</i>	phenazine biosynthesis protein	6.25	2.64	1.21E-18	1.36E-17
PA14_09480	<i>phzA1</i>	phenazine biosynthesis protein	6.92	2.79	2.12E-74	1.95E-72
PA14_09490	<i>phzM</i>	phenazine-specific methyltransferase	7.15	2.84	1.57E-79	1.68E-77
Type III secretion system						
PA14_00560	<i>exoT</i>	exoenzyme T	4.26	2.09	1.84E-23	2.70E-22
PA14_36345	<i>exoY</i>	adenylate cyclase	7.12	2.83	6.08E-45	2.37E-43
PA14_42430	<i>exsC</i>	exoenzyme S synthesis protein C	8.33	3.06	2.02E-23	2.95E-22
PA14_40290	<i>lasA</i>	LasA protease	4.94	2.31	1.63E-50	7.48E-49
Transcriptional regulators						
PA14_13150		transcriptional regulator	7.12	2.83	1.13E-71	9.55E-70
PA14_25800		TetR family transcriptional regulator	4.66	2.22	1.12E-41	3.85E-40

PA14_37990		RNA polymerase sigma factor	4.08	2.03	5.18E-28	9.48E-27
PA14_47580		MarR family transcriptional regulator	7.68	2.94	1.42E-33	3.48E-32
PA14_55160	<i>toxR</i>	ToxR transcriptional regulator	5.92	2.57	3.58E-32	8.07E-31
PA14_64700		RNA polymerase sigma factor	5.10	2.35	9.66E-51	4.49E-49
PA14_71070		AraC family transcriptional regulator	4.10	2.04	1.25E-30	2.73E-29
ECF sigma factors						
PA14_01840		RNA polymerase ECF-subfamily sigma-70 factor	7.08	2.82	1.78E-52	8.83E-51
PA14_32710		ECF subfamily RNA polymerase sigma-70 factor	6.44	2.69	7.10E-66	5.17E-64
PA14_33260	<i>pvdS</i>	extracytoplasmic-function sigma-70 factor	9.30	3.22	7.58E-62	5.01E-60
PA14_39800		ECF subfamily RNA polymerase sigma-70 factor	5.85	2.55	9.69E-51	4.49E-49
PA14_46660		RNA polymerase ECF-subfamily sigma-70 factor	8.26	3.05	6.46E-69	5.08E-67
PA14_47400		RNA polymerase ECF-subfamily sigma-70 factor	4.39	2.13	1.40E-39	4.41E-38
Glycerol metabolism						
PA14_38930	<i>glpR</i>	transcriptional regulator	11.51	3.53	1.60E-100	2.35E-98
Quorum sensing						
PA14_39980	<i>qscR</i>	transcriptional regulator	9.23	3.21	1.27E-78	1.33E-76
Translation, post-translational modification, degradation						
PA14_13840		peptidyl-prolyl cis-trans isomerase, PpiC-type	4.42	2.14	9.55E-34	2.36E-32
PA14_57580	<i>rpsI</i>	30S ribosomal protein S9	4.65	2.22	1.01E-25	1.67E-24
PA14_57590	<i>rplM</i>	50S ribosomal protein L13	4.36	2.12	2.40E-45	9.61E-44
PA14_60400	<i>rpsT</i>	30S ribosomal protein S20	5.16	2.37	5.28E-28	9.60E-27
Transport of small molecules						
PA14_01860		transmembrane sensor	6.10	2.61	1.15E-19	1.37E-18
PA14_02640	<i>mcdM</i>	malonate transporter subunit MadM	4.57	2.19	4.16E-13	3.19E-12
PA14_02690		amino acid permease	5.39	2.43	5.87E-15	5.15E-14
PA14_02700		aminotransferase	26.02	4.70	2.85E-35	7.42E-34
PA14_02720		hypothetical protein	40.01	5.32	2.56E-44	9.87E-43
PA14_16410		MFS transporter	11.10	3.47	1.05E-81	1.17E-79
PA14_18680	<i>bfd</i>	hypothetical protein	3.89	1.96	2.89E-07	1.26E-06
PA14_30590		outer membrane receptor protein	4.51	2.17	6.80E-19	7.76E-18
PA14_30600		permease	9.36	3.23	1.69E-46	6.96E-45
PA14_34770		ABC transporter substrate-binding protein	8.23	3.04	3.70E-11	2.37E-10
PA14_34780		ABC transporter ATP-binding protein	5.27	2.40	6.47E-24	9.79E-23
PA14_34790		ABC transporter permease	5.17	2.37	3.46E-14	2.85E-13
PA14_38220		hypothetical protein	4.47	2.16	4.86E-43	1.78E-41
PA14_38320	<i>yfeH</i>	bile acid/Na ⁺ symporter family transporter	8.67	3.12	5.06E-77	5.05E-75

PA14_38580		hypothetical protein	6.07	2.60	2.37E-23	3.43E-22
PA14_45240		amino acid permease	4.15	2.05	9.01E-16	8.43E-15
PA14_46910	<i>ybeJ</i>	ABC transporter substrate-binding protein	4.26	2.09	5.96E-40	1.90E-38
PA14_51880	<i>oprD</i>	basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor	5.27	2.40	2.47E-56	1.43E-54
PA14_53050	<i>aroP2</i>	aromatic amino acid transport protein AroP2	6.87	2.78	3.05E-69	2.42E-67
PA14_57990		hypothetical protein	5.29	2.40	3.89E-20	4.75E-19
PA14_64320		hypothetical protein	4.73	2.24	1.64E-22	2.25E-21
PA14_67840		ABC-type amino acid transporter	4.30	2.10	1.78E-20	2.21E-19
PA14_67860		ABC-type amino acid transporter	5.01	2.32	4.73E-23	6.75E-22
PA14_68070		periplasmic binding protein	5.18	2.37	1.80E-07	8.05E-07
PA14_72340	<i>gltP</i>	glutamate/aspartate:proton symporter	5.32	2.41	5.06E-23	7.19E-22
PA14_72710		transporter	3.91	1.97	1.08E-23	1.60E-22
PA14_73110		hypothetical protein	9.70	3.28	3.51E-16	3.34E-15
PA14_73120		hypothetical protein	4.55	2.19	3.82E-43	1.41E-41
Branched-chain amino acid transport						
PA14_50540	<i>livM</i>	leucine/isoleucine/valine transporter permease subunit	4.32	2.11	1.02E-27	1.83E-26
PA14_50550	<i>livG</i>	leucine/isoleucine/valine transporter ATP-binding subunit	4.75	2.25	1.09E-29	2.22E-28
PA14_64860	<i>livF</i>	ABC transporter ATP-binding protein	13.13	3.71	3.40E-19	3.95E-18
PA14_64870	<i>braF</i>	ABC transporter ATP-binding protein	12.53	3.65	6.23E-43	2.25E-41
PA14_64880	<i>braE</i>	branched chain amino acid ABC transporter permease	17.08	4.09	2.14E-48	9.21E-47
PA14_64890	<i>braD</i>	branched chain amino acid ABC transporter permease	6.71	2.75	1.18E-25	1.95E-24
C4-dicarboxylate transport						
PA14_52820	<i>dctQ</i>	C4-dicarboxylate transporter	27.85	4.80	3.01E-27	5.31E-26
PA14_52840	<i>dctP</i>	C4-dicarboxylate-binding periplasmic protein	15.64	3.97	1.45E-75	1.40E-73
Iron uptake						
PA14_02410		TonB-dependent receptor	36.54	5.19	2.79E-93	3.82E-91
PA14_02490	<i>tonB2</i>	hypothetical protein	15.54	3.96	3.57E-122	7.02E-120
PA14_02500	<i>exbB1</i>	transport protein ExbB	21.18	4.40	2.75E-153	8.11E-151
PA14_02510	<i>exbD1</i>	transport protein ExbD	26.20	4.71	1.09E-174	4.57E-172
PA14_09970	<i>fpvB</i>	type I ferripyoverdine receptor, FpvB	9.98	3.32	1.80E-87	2.26E-85
PA14_33680	<i>fpvA</i>	ferripyoverdine receptor	10.23	3.35	2.37E-106	3.78E-104
PA14_37980		Fe ²⁺ -dicitrate sensor, membrane protein	4.60	2.20	2.72E-12	1.96E-11
PA14_39820	<i>ufrA</i>	tonB-dependent receptor protein	5.75	2.52	1.34E-30	2.90E-29
PA14_45340		heme exporter protein	5.47	2.45	2.67E-30	5.74E-29

PA14_47380	<i>hxC</i>	heme utilization protein	4.40	2.14	4.31E-39	1.32E-37
PA14_68900	<i>fbpA</i>	iron ABC transporter substrate-binding protein	4.38	2.13	7.12E-20	8.63E-19
PA14_72640		TonB-dependent receptor	4.29	2.10	5.84E-40	1.87E-38
Pyochelin biosynthesis						
PA14_09270	<i>pchE</i>	dihydroaeruginoic acid synthetase	4.03	2.01	2.98E-12	2.14E-11
PA14_09280	<i>pchF</i>	pyochelin synthetase	4.00	2.00	5.59E-17	5.68E-16
Sulfonate transport						
PA14_02330	<i>atsC</i>	ABC transporter ATP- binding protein	29.85	4.90	4.46E-90	5.84E-88
PA14_02340	<i>atsB</i>	ABC transporter permease	25.73	4.69	7.96E- 151	2.04E- 148
PA14_02360	<i>atsR</i>	ABC transporter substrate-binding protein	14.31	3.84	1.58E-38	4.76E-37
PA14_12940		taurine ABC transporter ATP-binding protein	5.87	2.55	6.51E-29	1.28E-27
PA14_19510	<i>ssuC</i>	ABC transporter permease	4.67	2.22	3.26E-06	1.26E-05
PA14_19580	<i>ssuB</i>	aliphatic sulfonates transport ATP-binding subunit	7.35	2.88	4.94E-54	2.67E-52
Two-component regulatory systems						
PA14_06170	<i>fiuR</i>	transmembrane sensor	6.41	2.68	2.60E-59	1.60E-57
PA14_38740		two-component sensor	12.02	3.59	6.92E- 107	1.13E- 104
PA14_47390		transmembrane sensor	6.50	2.70	1.29E-65	9.15E-64
PA14_52250	<i>pirR</i>	two-component response regulator	3.91	1.97	6.14E-35	1.59E-33
PA14_57140		two-component response regulator	4.13	2.05	2.30E-20	2.84E-19
Chemotaxis and motility						
PA14_02250	<i>cheA</i>	two-component sensor	11.17	3.48	2.18E-94	3.14E-92
PA14_05330	<i>pilH</i>	twitching motility protein PilH	6.13	2.62	7.45E-55	4.14E-53
Ethanol catabolism						
PA14_38900	<i>exaE</i>	two-component response regulator	42.47	5.41	4.03E- 157	1.32E- 154
PA14_38910	<i>exaD</i>	sensor kinase	27.54	4.78	1.08E-91	1.45E-89
PA14_38970		two-component sensor	39.20	5.29	9.24E-61	5.91E-59
C4-dicarboxylate transport						
PA14_55780		two-component sensor	17.99	4.17	5.12E-48	2.17E-46

^a If part of an affected pathway or operon, genes differently expressed at least 1.5-fold were also considered.

^{b,c,d} Functional categorization, gene ID and gene/product names are taken from the Pseudomonas Genome database⁵² and KEGG (Kyoto Encyclopedia of Genes and Genome)⁵³.

180 b Transcripts with decreased abundance in B-96 cultures of PA14 Δhfq when compared with
 181 PA14. A fold change cut-off of -4 and a p-value above 0.05 were applied. The colour coding
 182 of different functional classes corresponds to those shown in Fig. 2b.

PA14_id ^b	Gene ^c	Description ^d	Fold Change	log2Fold Change	pval	padj
Adaptation, Protection						
PA14_21760	<i>capB</i>	cold acclimation protein B	-4.63	-2.21	4.69E-53	2.40E-51
PA14_27520	<i>gpo</i>	glutathione peroxidase	-4.53	-2.18	2.19E-38	6.59E-37
PA14_00710	<i>osmC</i>	osmotically inducible protein OsmC	-3.31	-1.72	2.62E-06	1.02E-05
PA14_64480	<i>osmE</i>	DNA-binding transcriptional activator OsmE	-2.34	-1.23	2.34E-08	1.14E-07
Amino acid biosynthesis and metabolism						
PA14_02730		dihydrodipicolinate synthetase	-36.75	-5.20	2.23E-75	2.12E-73
PA14_14020		hypothetical protein	-8.35	-3.06	7.39E-42	2.57E-40
PA14_39590	<i>metE</i>	5-methyltetrahydropteroyltriglutamate/homocysteine S-methyltransferase	-19.39	-4.28	7.81E-164	2.88E-161
Histidine degradation						
PA14_67320	<i>hutH</i>	histidine ammonia-lyase	-5.71	-2.51	8.93E-39	2.71E-37
PA14_67350	<i>hutU</i>	urocanate hydratase	-4.43	-2.15	8.95E-33	2.10E-31
Anthranilate biosynthesis						
PA14_51350	<i>phnB</i>	anthranilate synthase component II	-5.38	-2.43	4.48E-45	1.76E-43
PA14_51360	<i>phnA</i>	anthranilate synthase component I	-3.92	-1.97	8.87E-27	1.53E-25
Biosynthesis of cofactors, prosthetic groups and carriers						
PA14_01430		short chain dehydrogenase	-6.31	-2.66	4.78E-04	1.35E-03
PA14_06890		hypothetical protein	-5.03	-2.33	9.86E-36	2.62E-34
PA14_39640	<i>cobN</i>	cobaltochelatase subunit CobN	-13.18	-3.72	3.12E-06	1.20E-05
PA14_72980		G3E family GTPase	-6.87	-2.78	1.67E-05	5.92E-05
PA14_73010		hypothetical protein	-14.75	-3.88	1.59E-09	8.75E-09
Carbon compound catabolism						
PA14_14300		zinc-binding oxidoreductase	-6.30	-2.66	1.35E-04	4.23E-04
PA14_26130	<i>morB</i>	morphinone reductase	-4.74	-2.24	2.46E-42	8.61E-41
PA14_37770		hydrolase	-7.66	-2.94	8.92E-61	5.78E-59
PA14_54000	<i>prpD</i>	2-methylcitrate dehydratase	-4.42	-2.14	6.58E-11	4.14E-10
Cell wall / LPS / capsule						
PA14_73040	<i>amiA</i>	N-acetylmuramoyl-L-alanine amidase	-11.86	-3.57	9.89E-78	1.02E-75
PA14_61650	<i>pagL</i>	Lipid A 3-O-deacylase	-3.10	-1.63	7.98E-22	1.06E-20
PA14_17220	<i>lpxB</i>	lipid-A-disaccharide synthase	-2.58	-1.37	9.88E-16	9.19E-15
PA14_20890	<i>rfaD</i>	ADP-L-glycero-D-manno-heptose-6-epimerase	-2.31	-1.21	1.32E-09	7.36E-09
PA14_23380	<i>orfH</i>	UDP-N-acetyl-D-mannosaminuronate dehydrogenase	-1.68	-0.75	7.95E-05	2.56E-04

PA14_23420		zinc-binding dehydrogenase involved in O antigen biosynthesis	-2.72	-1.44	1.53E-02	3.21E-02
PA14_23430		heparinase	-2.47	-1.31	4.04E-02	7.57E-02
PA14_23450	<i>orfM</i>	NAD dependent epimerase/dehydratase	-1.68	-0.75	3.05E-02	5.95E-02
PA14_53500		predicted outer membrane protein and related peptidoglycan-associated (lipo)proteins	-2.14	-1.10	7.80E-11	4.88E-10
PA14_57370	<i>murD</i>	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	-2.53	-1.34	6.91E-12	4.79E-11
PA14_57390	<i>murF</i>	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase	-2.40	-1.27	1.15E-10	7.10E-10
PA14_57425	<i>ftsI</i>	penicillin-binding protein 3	-2.12	-1.08	1.31E-10	8.01E-10
PA14_57450	<i>mraW</i>	S-adenosyl-methyltransferase MraW	-2.21	-1.14	3.09E-10	1.84E-09
PA14_62840	<i>glmM</i>	phosphoglucosamine mutase	-2.04	-1.03	1.25E-11	8.44E-11
PA14_68170	<i>rmlB</i>	dTDP-D-glucose 4,6-dehydratase	-3.38	-1.76	3.53E-10	2.09E-09
PA14_68210	<i>rmlC</i>	dTDP-4-dehydrorhamnose 3,5-epimerase	-2.61	-1.39	4.23E-18	4.56E-17
PA14_23460	<i>orfN</i>	group 4 glycosyl transferase	-1.78	-0.83	1.59E-02	3.34E-02
Central intermediary metabolism						
PA14_45000	<i>gcl</i>	glyoxylate carboligase	-17.13	-4.10	1.34E-122	2.73E-120
PA14_45010	<i>hyi</i>	hydroxypyruvate isomerase	-7.79	-2.96	6.89E-24	1.04E-22
Chaperones & heat shock proteins						
PA14_23680	<i>ibpA</i>	heat-shock protein IbpA	-4.25	-2.09	4.04E-25	6.44E-24
PA14_42980		ClpA/B-type protease	-9.94	-3.31	4.27E-39	1.32E-37
PA14_62960	<i>dnaJ</i>	chaperone protein DnaJ	-3.97	-1.99	1.45E-09	7.98E-09
PA14_62970	<i>dnaK</i>	molecular chaperone DnaK	-4.63	-2.21	1.31E-48	5.73E-47
PA14_62990	<i>grpE</i>	heat shock protein GrpE	-4.05	-2.02	3.73E-21	4.80E-20
PA14_60190	<i>clpB</i>	clpB protein; ATP-binding subunits of Clp protease and DnaK/DnaJ chaperones	-2.82	-1.49	2.85E-22	3.86E-21
PA14_54210	<i>asrA</i>	ATP-dependent protease; aminoglycoside-induced stress response ATP-dependent protease AsrA; protein turnover	-2.48	-1.31	4.20E-08	2.00E-07
Chemotaxis						
PA14_46030		chemotaxis transducer	-4.97	-2.31	1.41E-27	2.52E-26
DNA replication, recombination, modification and repair						
PA14_30160	<i>mutT</i>	hypothetical protein	-8.07	-3.01	1.00E-29	2.07E-28
Energy metabolism						
PA14_05820		hypothetical protein	-5.85	-2.55	4.04E-11	2.58E-10
PA14_06960		hypothetical protein	-11.83	-3.56	1.86E-93	2.60E-91
PA14_63100		ferredoxin	-8.19	-3.03	2.80E-74	2.54E-72
Glycerol metabolism						
PA14_17930	<i>glpD</i>	glycerol-3-phosphate dehydrogenase	-4.84	-2.27	1.22E-25	2.00E-24

TCA cycle

PA14_30180	<i>idh</i>	monomeric isocitrate dehydrogenase	-5.22	-2.38	3.51E-28	6.50E-27
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Lactate oxidation

PA14_33860	<i>lldA</i>	L-lactate dehydrogenase	-6.22	-2.64	6.10E-29	1.20E-27
PA14_63090	<i>lldD</i>	L-lactate dehydrogenase	-10.39	-3.38	9.63E-70	7.88E-68

Denitrification (*nuo* operon; NADH dehydrogenase)

PA14_30020	<i>nuoA</i>	NADH dehydrogenase subunit A	-4.48	-2.16	2.27E-35	5.93E-34
PA14_29900	<i>nuoJ</i>	NADH dehydrogenase subunit J; NADH-quinone oxidoreductase subunit J	-2.38	-1.25	2.85E-06	1.11E-05
PA14_29920	<i>nuoI</i>	NADH dehydrogenase subunit I; NADH-quinone oxidoreductase subunit I	-2.41	-1.27	1.62E-08	7.99E-08
PA14_29930	<i>nuoH</i>	NADH dehydrogenase subunit H; NADH-quinone oxidoreductase subunit H	-2.43	-1.28	4.29E-13	3.28E-12
PA14_29970	<i>nuoF</i>	NADH dehydrogenase subunit F; NADH-quinone oxidoreductase subunit F	-3.20	-1.68	3.42E-26	5.72E-25
PA14_29940	<i>nuoG</i>	NADH dehydrogenase subunit G; NADH-quinone oxidoreductase subunit G	-2.20	-1.14	5.40E-14	4.41E-13
PA14_29980	<i>nuoE</i>	NADH dehydrogenase subunit E; NADH-quinone oxidoreductase subunit E	-2.91	-1.54	2.11E-20	2.61E-19
PA14_29990	<i>nuoD</i>	bifunctional NADH:ubiquinone oxidoreductase subunit C/D	-3.33	-1.74	8.43E-30	1.76E-28
PA14_30010	<i>nuoB</i>	NADH dehydrogenase subunit B; NADH-quinone oxidoreductase subunit B	-2.80	-1.49	8.45E-23	1.18E-21

Fatty acid and phospholipid metabolism

PA14_05840	<i>gcdH</i>	glutaryl-CoA dehydrogenase	-10.89	-3.45	1.47E-09	8.08E-09
PA14_11280		hypothetical protein	-13.31	-3.73	3.09E-20	3.78E-19
PA14_16640		lipoprotein	-6.08	-2.60	3.20E-18	3.48E-17
PA14_40800		hypothetical protein	-6.22	-2.64	7.65E-34	1.90E-32
PA14_71890	<i>psecoA</i>	coenzyme A transferase	-9.01	-3.17	3.71E-66	2.73E-64

Hypothetical, unclassified, unknown

PA14_06040		hypothetical protein	-4.21	-2.07	3.49E-24	5.35E-23
PA14_10360		hypothetical protein	-5.39	-2.43	4.15E-45	1.64E-43
PA14_11320		hypothetical protein	-15.46	-3.95	3.89E-18	4.20E-17
PA14_12530		hypothetical protein	-5.58	-2.48	9.57E-44	3.57E-42
PA14_15710		hypothetical protein	-4.32	-2.11	4.42E-33	1.05E-31
PA14_16990		hypothetical protein	-12.86	-3.68	3.12E-119	5.74E-117
PA14_18960		hypothetical protein	-5.05	-2.33	6.74E-24	1.02E-22
PA14_28360		hypothetical protein	-6.60	-2.72	3.59E-56	2.05E-54
PA14_31420		hypothetical protein	-5.27	-2.40	1.06E-47	4.47E-46
PA14_32780		hypothetical protein	-6.98	-2.80	9.80E-29	1.89E-27
PA14_37780		hypothetical protein	-11.28	-3.50	6.86E-85	8.42E-83
PA14_38270		hypothetical protein	-4.37	-2.13	2.83E-30	6.03E-29
PA14_39660		hypothetical protein	-8.30	-3.05	5.44E-25	8.61E-24
PA14_43050		hypothetical protein	-5.98	-2.58	1.64E-64	1.12E-62
PA14_44990		hypothetical protein	-6.82	-2.77	3.85E-41	1.30E-39

PA14_47130		hypothetical protein	-5.50	-2.46	2.31E-26	3.92E-25
PA14_53980		hypothetical protein	-5.63	-2.49	1.97E-40	6.44E-39
PA14_54180		hypothetical protein	-26.04	-4.70	3.68E-28	6.78E-27
PA14_56170		hypothetical protein	-7.94	-2.99	9.94E-13	7.36E-12
PA14_58230		hypothetical protein	-6.66	-2.74	3.43E-14	2.83E-13
PA14_58240		hypothetical protein	-4.82	-2.27	5.47E-23	7.71E-22
PA14_60540		hypothetical protein	-3.92	-1.97	4.12E-10	2.43E-09
PA14_60650		hypothetical protein	-5.03	-2.33	3.37E-31	7.45E-30
PA14_63920		hypothetical protein	-48.89	-5.61	1.25E-20	1.56E-19
PA14_63940		hypothetical protein	-61.79	-5.95	1.08E-13	8.59E-13
PA14_68840		hypothetical protein	-5.61	-2.49	5.61E-38	1.65E-36
PA14_73000		hypothetical protein	-19.64	-4.30	3.26E-14	2.69E-13
PA14_73050		GTP cyclohydrolase	-12.25	-3.62	1.01E-28	1.94E-27
Membrane proteins						
PA14_00510		hemagglutinin	-4.69	-2.23	2.12E-40	6.87E-39
PA14_11290		permease	-11.02	-3.46	3.04E-34	7.65E-33
PA14_16630		outer membrane protein, OmpA	-5.22	-2.38	3.61E-29	7.21E-28
PA14_18720	<i>motY</i>	OmpA family membrane protein	-4.15	-2.05	1.59E-23	2.35E-22
PA14_26420		TonB-dependent receptor	-4.02	-2.01	3.87E-05	1.31E-04
PA14_54520	<i>opdH</i>	porin	-3.90	-1.96	1.13E-14	9.69E-14
PA14_60630		hypothetical protein	-5.29	-2.40	5.29E-35	1.37E-33
PA14_63910		hypothetical protein	-43.91	-5.46	3.51E-24	5.38E-23
PA14_63960		uter membrane protein	-31.62	-4.98	5.17E-23	7.31E-22
PA14_63970		hypothetical protein	-13.76	-3.78	2.20E-13	1.72E-12
Nucleotide biosynthesis and metabolism						
PA14_73070	<i>pyrQ</i>	dihydroorotase	-16.06	-4.01	2.60E-17	2.70E-16
Protein secretion/export apparatus						
PA14_10340	<i>cvaB</i>	toxin transporter	-4.21	-2.07	3.61E-12	2.58E-11
PA14_10350		secretion protein	-6.81	-2.77	1.97E-18	2.17E-17
Type VI secretion system						
PA14_42950		predicted type VI secretion system protein ImpI	-8.75	-3.13	2.32E-10	1.39E-09
PA14_42990		predicted type VI secretion system protein ImpH	-6.28	-2.65	2.70E-41	9.19E-40
PA14_43000		predicted type VI secretion system protein ImpG	-10.94	-3.45	2.67E-66	1.99E-64
PA14_43020		predicted type VI secretion system protein	-6.97	-2.80	2.64E-15	2.37E-14
PA14_43030		predicted type VI secretion system protein ImpC	-7.29	-2.87	5.20E-30	1.10E-28
PA14_43040		predicted type VI secretion system protein ImpB	-5.47	-2.45	5.36E-43	1.95E-41
Putative enzymes						
PA14_09580		hypothetical protein	-6.36	-2.67	7.19E-44	2.73E-42
PA14_09950		oxidoreductase	-8.62	-3.11	8.50E-67	6.50E-65
PA14_17730		hypothetical protein	-8.02	-3.00	5.43E-37	1.51E-35
PA14_37745		carbamoyl transferase	-5.28	-2.40	8.02E-53	4.04E-51
PA14_45020	<i>glxR</i>	oxidoreductase	-5.32	-2.41	1.05E-08	5.26E-08
PA14_46620		pyridine nucleotide-disulfide oxidoreductase	-4.55	-2.19	4.71E-44	1.80E-42

PA14_49800	<i>gsp69</i>	oxidoreductase	-5.14	-2.36	3.25E-27	5.71E-26
PA14_56160	<i>moxR</i>	hypothetical protein	-4.09	-2.03	4.52E-23	6.46E-22
Secreted Factors (toxins, enzymes, alginate)						
Rhamnolipids biosynthesis						
PA14_19100	<i>rhlA</i>	rhamnosyltransferase chain A	-4.97	-2.31	1.03E-53	5.48E-52
PA14_19110	<i>rhlB</i>	rhamnosyltransferase chain B	-3.60	-1.85	3.67E-11	2.36E-10
Transcription, RNA processing and degradation						
PA14_08760	<i>rpoB</i>	DNA-directed RNA polymerase subunit beta	-4.12	-2.04	6.61E-46	2.67E-44
PA14_08780	<i>rpoC</i>	DNA-directed RNA polymerase subunit beta'	-9.68	-3.28	3.83E-12	2.73E-11
Transcriptional regulators						
PA14_00460	<i>trpI</i>	transcriptional regulator TrpI	-4.76	-2.25	8.13E-17	8.17E-16
PA14_01640		LysR family transcriptional regulator	-4.67	-2.22	1.69E-22	2.32E-21
PA14_06970		Cro/CI family transcriptional regulator	-4.84	-2.27	1.25E-29	2.55E-28
PA14_09570		LysR family transcriptional regulator	-4.98	-2.32	7.26E-32	1.63E-30
PA14_23730		LysR family transcriptional regulator	-6.60	-2.72	5.01E-30	1.07E-28
PA14_34880		GntR family transcriptional regulator	-5.54	-2.47	1.89E-49	8.42E-48
PA14_38680		LysR family transcriptional regulator	-6.15	-2.62	8.94E-36	2.39E-34
PA14_42970		transcriptional regulator	-10.50	-3.39	2.19E-12	1.59E-11
PA14_46480		transcriptional regulator	-6.06	-2.60	1.49E-35	3.92E-34
PA14_47520		transcriptional regulator	-6.12	-2.61	1.56E-40	5.16E-39
Quorum sensing						
PA14_45960	<i>lasR</i>	transcriptional regulator LasR; quorum sensing	-1.31	-0.39	1.48E-02	3.12E-02
PA14_19120	<i>rhlR</i>	transcriptional regulator RhlR; quorum sensing	-2.29	-1.19	1.68E-16	1.65E-15
Denitrification						
PA14_29620	<i>norR</i>	anaerobic nitric oxide reductase transcriptional regulator; activator; denitrification	-2.45	-1.29	8.47E-14	6.83E-13
Translation, post-translational modification, degradation						
PA14_17700	<i>rpmE2</i>	50S ribosomal protein L31	-9.38	-3.23	2.56E-15	2.31E-14
PA14_17710	<i>rpmJ</i>	50S ribosomal protein L36	-7.44	-2.89	1.50E-33	3.65E-32
Transport of small molecules						
PA14_11310		ABC transporter ATP-binding protein	-11.66	-3.54	4.74E-18	5.09E-17
PA14_13990		amino acid ABC transporter	-5.31	-2.41	6.03E-53	3.06E-51
PA14_15200		hypothetical protein	-7.59	-2.92	3.02E-34	7.64E-33
PA14_15700		amino acid permease	-4.25	-2.09	2.71E-33	6.52E-32
PA14_18670	<i>bfrB</i>	bacterioferritin	-5.62	-2.49	5.45E-56	3.09E-54
PA14_22650		ABC transporter	-6.08	-2.60	1.44E-25	2.36E-24
PA14_24830		glutathione S-transferase	-8.39	-3.07	4.19E-38	1.24E-36
PA14_37760		MFS transporter	-6.43	-2.68	2.13E-54	1.17E-52
PA14_39650	<i>cirA</i>	TonB-dependent receptor	-7.73	-2.95	2.91E-19	3.41E-18
PA14_44520	<i>ydgF</i>	drug efflux transporter	-25.66	-4.68	2.54E-151	6.80E-149
PA14_44530	<i>ygdE</i>	multidrug efflux system protein MdtI	-9.50	-3.25	3.29E-64	2.23E-62

PA14_44820		transthyretin family protein	-8.54	-3.10	3.87E-77	3.94E-75
PA14_44950	<i>ygfU</i>	transporter	-10.28	-3.36	2.42E-73	2.16E-71
PA14_49130	<i>dctA</i>	C4-dicarboxylate transporter DctA	-3.93	-1.97	6.88E-19	7.84E-18
PA14_63080	<i>lldP</i>	L-lactate permease	-6.45	-2.69	1.56E-56	9.09E-55
PA14_67340	<i>codB</i>	cytosine/purines uracil thiamine allantoin permease	-5.77	-2.53	1.28E-29	2.60E-28
PA14_73060	<i>cynT</i>	hypothetical protein	-13.23	-3.73	1.77E-17	1.85E-16
Two-component regulatory systems						
PA14_21700		two-component sensor	-5.35	-2.42	4.55E-17	4.66E-16
PA14_64410	<i>ybiI</i>	hypothetical protein	-9.85	-3.30	1.29E-22	1.78E-21
PA14_73020		DksA/TraR family C4-type zinc finger protein	-21.61	-4.43	3.97E-13	3.04E-12

^a If part of an affected pathway or operon, genes differently expressed at least 1.5-fold were also considered.

^{b,c,d} Functional categorization, gene ID and gene/product names are taken from the *Pseudomonas* Genome database⁵² and KEGG (Kyoto Encyclopedia of Genes and Genome)⁵³.

52. Winsor, G. L. *et al.* Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res.* **44**, D646-53 (2016).

53. Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M. & Tanabe, M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* **44**, D457-62 (2016).

194 **Supplementary Table S3. Strains and plasmids used in this study.**

Strain/plasmid	Genotype/relevant features	Source/reference ^a
<i>P. aeruginosa</i>		
PA14	Wild-type	57
PA14 <i>anr</i> -	Transposon insertion in <i>anr</i> ; library ID : 26855	57
PA14 <i>rpoN</i> -	Transposon insertion in <i>rpoN</i> ; library ID :44482	57
PA14Δ <i>hfq</i>	In-frame deletion of the <i>hfq</i> gene	58
PA14Δ <i>crcZ</i>	In-frame deletion of the <i>crcZ</i> gene	This study
PA14Δ <i>hfq</i> Δ <i>crcZ</i>	Deletion of the <i>hfq</i> - and of the <i>crcZ</i> genes	This study
<i>E. coli</i>		
DH5α	<i>F</i> ⁻ , <i>φ80dlacZ</i> Δ <i>M15</i> , Δ(<i>lacZYA</i> - <i>argF</i>) <i>U169</i> , <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (<i>rk</i> ⁻ , <i>mk</i> ⁺), <i>phoA</i> , <i>supE44</i> , <i>λ</i> ⁻ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Invitrogen
HB101	<i>thi-1 hsdS20</i> (<i>rB</i> ⁻ , <i>mB</i> ⁻) <i>supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20</i>	59
Plasmids		
pME3087	Suicide vector, ColE1 replicon, IncP-1, Mob; Tc ^R	60
pME3087Δ <i>crcZ</i>	Suicide vector used for construction of the in-frame deletion of <i>crcZ</i> ; Tc ^R	This study
pME3087Δ <i>hfq</i>	Suicide vector used for construction of the in-frame deletion of <i>hfq</i> ; Tc ^R	This study
pRK2013	Helper plasmid, ColE1 replicon, Tra; Km ^r	61
pUX-BF13	<i>mob</i> ⁺ <i>ori</i> -R6K; helper plasmid, providing the Tn7 transposition functions <i>in trans</i> ; Amp ^r	62
pBK-mini-Tn7- <i>rrnBP1</i> - <i>gfp</i> (AGA)	Delivery plasmid for mini-Tn7- <i>rrnBP1</i> - <i>gfp</i> -AGA-a, Amp ^r , Gm ^r	24
pMMB <i>crcZ</i>	pMMBΔ <i>rbs</i> harboring <i>crcZ</i> under transcriptional control of P _{tac}	15
^a Additional references:		
57.	Liberati, N. T. et al. An ordered, nonredundant library of <i>Pseudomonas aeruginosa</i> strain PA14 transposon insertion mutants. <i>Proc. Natl. Acad. Sci. United States Am.</i> 103, 2833–2838 (2006).	
58.	Wurtzel, O. et al. The single-nucleotide resolution transcriptome of <i>Pseudomonas aeruginosa</i> grown in body temperature. <i>PLoS Pathog.</i> 8, e1002945 (2012).	
59.	Boyer, H. W. & Roulland-Dussoix, D. A complementation analysis of the restriction and modification of DNA in <i>Escherichia coli</i> . <i>J. Mol. Biol.</i> 41, 459–472 (1969).	
60.	Voisard, C. et al. in <i>Molecular Ecology of Rhizosphere Microorganisms</i> 67–89 (Wiley-VCH Verlag GmbH, 1994). doi:10.1002/9783527615810.ch6	
61.	Figurski, D. H. & Helinski, D. R. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in <i>trans</i> . <i>Proc. Natl. Acad. Sci. U. S. A.</i> 76, 1648–1652 (1979).	
62.	Bao, Y., Lies, D. P., Fu, H. & Roberts, G. P. An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. <i>Gene</i> 109, 167–168 (1991).	

209 **Supplementary Table S4. Oligonucleotides used in this study.**

Name	Sequence ^a	Restriction site
C3	GCACAACAACAATAACAAGCAACGACGAAG	
C6	ATGCGGATCCGAAATGGTGTAAGGCGAAGG	<i>Bam</i> HI
D112	GTACAGGGAACACGCAACC	
E2	CTTCTTCCGACTGGCTGCGGG	
E6	TCTAGAC <u>GTAAATACGACTCACTATAGGC</u> CACAACAACAATAA CAAGC	<i>Xba</i> I
E112	CCCCGCCCCTCTTCAG	
H71	TTTTTTT GGATCC GATCCCAGGTGCGCGC	<i>Bam</i> HI
I26	CCCCACACTACCATCGGCGATGCGTCG	
I71	TTTTTTT GAATTC GCGGGCAGGTGCG	<i>Eco</i> RI
J71	GTGCCGCACTCCTTTAAGG	
K3	GCTGGAGTCGTTACGTGTTG	
K71	CCTTAAAGGAGTGCGGCACTGACGGGAGTCCGCTTTG	
T131	CGAATGGCTTCTTGAGC	
U131	GAGCTTCGTATGGGGAG	
W100	GCATAC GGTACC ACCGCGACCTGAAGACCCTGGC	<i>Kpn</i> I
X100	GCTCAT TCTAGAC AGTTTGAAGCGGCGGCCGATG	<i>Xba</i> I
Y100	CAATACATAAGCAGATGCCGTGCCAG	
Z100	CTGGCACGGCATCTGCTTATGTATTGAAACAAAGCACGCACCTACTTGGG	

^a Restriction sites are highlighted in bold. The T7 promoter sequence is underlined.

5.2 RpoN is required for full-fledged anoxic transcription of the anaerobic regulator gene *anr* in *Pseudomonas aeruginosa* O1

**RpoN is required for full-fledged anoxic transcription of the
anaerobic regulator gene *anr* in *Pseudomonas aeruginosa* O1**

Petra Pusic, Udo Bläsi* and Elisabeth Sonnleitner*

Department of Microbiology, Immunobiology and Genetics, Center of Molecular Biology, Max F.
Perutz Laboratories, University of Vienna, Vienna Biocenter, Dr. Bohrgasse 9/4, 1030 Vienna,
Austria

*Corresponding authors

Udo Bläsi

E-mail: udo.blaesi@univie.ac.at

Elisabeth Sonnleitner

E-mail: elisabeth.sonnleitner@univie.ac.at

21 **Summary**

22 The anaerobic regulator Anr is required for anaerobic respiration and anoxic biofilm formation
23 during chronic infections of the opportunistic human pathogen *Pseudomonas aeruginosa*. Here, we
24 describe a regulatory network composed of several sigma factors that govern transcription of the
25 *anr* gene. The study revealed that the sigma factor RpoN in conjunction with the transcription factor
26 NtrC is required for full-fledged anoxic transcription of *anr* in *P. aeruginosa* PAO1, while the
27 sigma factors RpoH and FliA are directly or indirectly contributing to residual *anr* promoter activity
28 during anaerobiosis.

29

30 **Introduction**

31 *Pseudomonas aeruginosa* is an opportunistic human pathogen that causes severe infections in
32 immunocompromised individuals, burn patients, and patients suffering from cystic fibrosis. *P.*
33 *aeruginosa* can form biofilms in the lungs of cystic fibrosis patients under microaerophilic or
34 anaerobic conditions, underscoring the importance of anaerobic metabolism for pathogenicity [1–3].

35 The switch of *P. aeruginosa* from aerobic to anaerobic growth is controlled by the master
36 regulator of anaerobic metabolism, Anr [4], a homologue of the *Escherichia coli* anaerobic
37 regulator Fnr [5]. The active enzymatic core of Anr accomodates iron-sulfur clusters that are
38 responsive to the low-oxygen tension. Under hypoxic conditions Anr forms active dimers that
39 recognize 5'..TTGATNNNNATCAA..3' motifs [6], whereas in the presence of oxygen, the iron-
40 sulfur clusters are damaged leading to inactivation of Anr [7]. Under hypoxic/anoxic conditions,
41 Anr acts as an upstream regulator of the denitrification pathway by activating transcription of the
42 nitrate-dependent two-component system NarXL and the nitric oxide-responsive regulator Dnr
43 [8,9]. In conjunction with NirQ, transcription of which is activated by Dnr, these transcription
44 factors control a regulatory cascade, which leads to reduction of nitrate to dinitrogen in four
45 consecutive steps (for review, [10]). A previous transcriptome analysis and a bioinformatic
46 approach identified 36 *P. aeruginosa* genes that are directly regulated by Anr and 130 additional
47 putative Anr binding sites, respectively [11]. Anr regulates genes required for anaerobic respiration
48 [12–14], synthesis of virulence factors [15,16], synthesis of haem [17,18], synthesis of stress
49 proteins [19–21], as well as the expression of the small RNA PhrS [22]. An *anr* deficient mutant of
50 strain *P. aeruginosa* 14 (PA14) was impaired in forming biofilm like structures in the
51 hypoxic/anaerobic necrotic centers of transplanted subcutaneous mouse tumors, underlining the
52 pivotal role of Anr in anoxic biofilm formation [23].

53 The sigma factor RpoN (σ^{54}) recognizes the consensus sequence 5'..TGGCAC-N₅-
54 TTGCW..3' (bold: invariant nucleotides, W = A or T) [24] and transcription requires at least one

transcriptional activator [25,26]. RpoN-dependent transcription involves bending of the DNA region between the activator binding site and the RpoN binding site to allow contact between the two protein complexes. In some cases, this bending is assisted by nucleoid-associated proteins, such as IHF or HU [27,28]. In general, transcriptional activators that control RpoN-dependent promoters employ ATP hydrolysis to enable transcription initiation [29]. In *Pseudomonas*, RpoN controls functions involved in motility, such as pilus and flagellum synthesis [30,31], metabolism [32], biofilm formation [33], quorum sensing (QS) and virulence [34,35]. Mutations in *rpoN* resulted in reduced virulence in several infection models, which was mainly attributed to defects in adhesion, motility and cell-to-cell communication [36].

Although a RpoN binding motif was identified upstream of the *anr* coding sequence (Fig 1), it remained unclear whether *anr* transcription is controlled by RpoN in *P. aeruginosa* K (PAK) [37]. More recently, chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) indicated that not only RpoN, but also the stress-related sigma factor RpoS and the motility sigma factor FliA bind to the *anr* promoter region of *P. aeruginosa* strain PA14 (Fig 1) [38]. The alternative sigma factor RpoS is a master regulator of the stress response and involved in regulation of QS [39], production and secretion of virulence factors [40–42] and biofilm formation [43]. The main function of sigma factor FliA is linked to the control of flagellin biosynthesis [36]. A Δ *fliA* strain was non-motile, as FliA is necessary for expression of the flagellin gene *fliC* [44]. FliC is required for type IV pili-mediated twitching motility and plays an important role in both aerobic and anoxic biofilm formation [1]. In addition to the transcriptional control of flagellar biosynthesis, FliA seems to control *P. aeruginosa* motility by modulating the c-di-GMP levels [45]. Moreover, our screen for regulatory promoter elements revealed a putative signature for RpoH (Fig 1), overlapping with the RpoN interaction site. *E. coli* RpoH is responsible for the heat-shock response [46], however, little is known about the RpoH regulon in *P. aeruginosa*. Recently, a study from Schulz *et al.* identified genes involved in adaptation and protection, heat-shock response,

80 motility and attachment and energy metabolism as important members of the RpoH regulon [38]. In
81 the present study, we have revisited *anr* transcription in *P. aeruginosa* O1 (PAO1) and show that
82 RpoN represents an major player within the regulatory network governing *anr* transcription during
83 anaerobiosis.

84

85 **Fig 1. Promoter region of the *anr* gene.** The sequence of the RpoN-dependent promoter is
86 highlighted in orange with the corresponding consensus sequence shown on top [24]. Mutations
87 introduced into the -24 and -12 box of the promoter region are indicated by black arrows. The
88 putative NtrC binding motif is depicted in green with the corresponding consensus sequence shown
89 on top [47]. The palindromic sequences of the NtrC binding site are indicated by arrows. The
90 deletion of the NtrC binding site present in plasmid pME6016anr5 is shaded. The predicted RpoH
91 binding site is underlined in yellow ochre and the corresponding *E. coli* consensus sequence [48] is
92 shown below. The sequence of the predicted FliA binding motif and a putative RpoS binding site
93 are indicated in red and violet, respectively, with the corresponding consensus motifs [38] shown on
94 top. The experimentally determined transcriptional start site of *anr* [49] is indicated as +1, the
95 upstream untranslated region is shown in blue and the beginning of the *anr* coding region is
96 highlighted by a blue arrow. Filled arrowheads denote the 5' and 3' boundaries of the DNA
97 segments present in the respective transcriptional *anr-lacZ* reporter genes. Altered nucleotides
98 within the PAO1 sequence when compared with PA14 and PAK are shown in dark red and brown,
99 respectively.

100 **Materials and methods**

101 **Bacterial strains and growth conditions**

102 The strains and plasmids used in this study are listed in S1 Table. The cell cultures were first grown
103 overnight in LB medium and washed with BSM minimal medium (30.8 mM K₂HPO₄, 19.3 mM
104 KH₂PO₄, 15 mM (NH₄)₂SO₄, 1 mM MgCl₂, 2 μM FeSO₄) supplemented with 40 mM succinate,
105 0.2% glutamine (to allow for growth of the glutamine auxotroph strain PAO1Δ*rpoN* [30] and 100
106 mM KNO₃ (for anaerobic nitrate respiration) (BSM_{anox} medium). The cells were then diluted to an
107 OD₆₀₀ of 0.1 and grown anaerobically at 37°C in an anaerobic chamber fumigated with N₂ (98%)
108 and H₂ (2%). The *E. coli* strain DH5α was used for the construction of plasmids. When required, the
109 following concentrations of antibiotics were used: 100 μg/ml ampicillin and 25 μg/ml tetracycline
110 for *E. coli*; 50 μg/ml gentamycin, 250 μg/ml carbenicillin and 100 μg/ml of tetracycline for *P.*
111 *aeruginosa*. The construction of plasmids used in this study is described in S1 Text.

112 **β-galactosidase assays**

113 The β-galactosidase assays were performed after anaerobic cultivation for 22 hours in BSM_{anox}
114 medium as previously described [50], except that the cells were permeabilized with 5% toluene and
115 the β-galactosidase activities were measured at 37°C. The Miller units shown in the respective
116 graphs are the mean values of at least three biological replicates. Error bars represent standard
117 deviation.

118 **RNA isolation and RT-PCR**

119 RNA was extracted from cells grown anaerobically in BSM_{anox} medium for 22 hours using the
120 innuSOLV RNA Reagent (Analytik Jena) according to the manufacturer's instructions. The samples
121 were TURBO DNase (Ambion) treated, followed by phenol-chloroform-isoamyl alcohol extraction

122 and ethanol precipitation. For cDNA synthesis, 250 ng RNA template was mixed with 125 ng of
123 random primers (Promega). The mixture was then treated at 70°C for 10 min, followed by 5 min
124 incubation on ice. The cDNA synthesis was performed with AMV reverse transcriptase (Promega)
125 according to the manufacturer's instructions. 4 µl of cDNA was further used as a template for PCR
126 amplification using the oligonucleotides I129 and J129 (S2 Table; *anr*) or Q117 and R117 (S2
127 Table; *rpoD*), respectively. The corresponding PCR products were 202 bp (*anr*) and 81 bp (*rpoD*)
128 in length. To confirm total DNA depletion of the RNA samples, the PCR reactions were performed
129 with the same amount of RNA as used for the RT-PCR reaction without reverse transcriptase.

130 **Biofilm assays**

131 Biofilms were established with 100 µl bacterial cultures grown in BSM_{anox} medium in µclear half-
132 area flat bottom 96-well black polystyrene plates (Greiner Bio-One, Germany). The initial OD₆₀₀
133 was set to 0.05 and the cultures were incubated for 48 hours in an anaerobic chamber at 37°C. The
134 biofilms were stained with SYTO 9 fluorescent dye (live cells) and propidium iodide (dead cells)
135 using the BacLight LIVE/DEAD viability kit according to the manufacturer's instructions
136 (Molecular Probes/ThermoFischer Scientific). Image acquisition was performed at the MFPL
137 BioOptics Facility with a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss,
138 Germany) equipped with 405, 488, 555 and 639 nm laser diodes and two PMT channels for
139 simultaneous monitoring of GFP (excitation, 488 nm; emission, 517 nm) and propidium iodide
140 (excitation, 543 nm; emission, 565 nm). Images were obtained using a Plan-Apochromat 40x/1.3
141 Oil DIC objective. Simulated multi-channel cross-sections were generated using Fiji: an open-
142 source platform for biological-image analysis and Volume Viewer Plugin [51]. The quantitative
143 analyses of acquired image stacks of biofilms were performed using Comstat2 software [52,53].

144

145 **Statistical analysis**

146 All experiments were performed at least in duplicate. The data shown were obtained with three
147 biological replicates, unless indicated otherwise. Statistical significance was determined by
148 unpaired two-tailed student *t*-test or one-way ANOVA with the Dunnett's multiple comparison test
149 and the Tukey's post hoc test (<https://www.graphpad.com>), respectively, when more than two
150 groups with normal distribution were compared. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and
151 *****P* < 0.0001 were considered statistically significant results.

152

153 **Results and Discussion**

154 **RpoN is a major regulator of *anr* transcription during anaerobiosis**

155 In contrast to *E. coli fnr*, no motifs for RpoD and sites for negative autoregulation could be
156 discerned upstream of the *anr* gene in *P. aeruginosa* [49]. Instead, a -24 (5'..TGGCAC..3') and -12
157 (5'..TCGC..3') sequence motif is present (Fig 1, in orange), which represents a putative signature
158 for RpoN-dependent promoters [24]. In addition, a putative binding site for the response regulator
159 NtrC [47] is present between nucleotides -124 to -108 upstream of the transcriptional start site of
160 *anr* (Fig 1, in green). A previous study indicated that *anr* transcription does not depend on RpoN in
161 *P. aeruginosa* strain PAK [37]. The *anr* promoter regions in PAO1 and the PAK differ by the T₋₁₀₆→
162 >A and G₋₂₈→A substitutions downstream of the NtrC binding site and within the putative RpoN
163 binding motif, respectively (Fig 1, in brown). Hence, it appears possible that these nucleotide
164 exchanges render the *anr* promoter in PAK independent of RpoN. However, ChIP-seq analyses
165 indicated an interaction of RpoN with the *anr* promoter in the *P. aeruginosa* strain PA14 [38]. The
166 *anr* promoter regions of PAO1 and PA14 are identical except for a single nucleotide exchange at
167 position -56 relative to the transcriptional start site (Fig 1, in dark red), which would not affect the
168 putative RpoN binding site.

169 To test whether the *anr* promoter activity depends on RpoN in PAO1, PAO1 wild-type (wt)
 170 and the isogenic *rpoN* deficient strain, respectively, were transformed with plasmid pME6016anr2
 171 harbouring a transcriptional *anr-lacZ* reporter gene (S1 Table and S1 Text). The cells were grown in
 172 BSM medium supplemented with 40 mM succinate, 0.2% glutamine and 100 mM KNO₃ (BSM_{anox}
 173 medium) in an anaerobic chamber for 22 hours, and the *anr* promoter activity in either strain was
 174 then monitored by measuring the β -galactosidase activities. As shown in Fig 2A, the *anr* promoter
 175 activity was ~3-fold decreased in the strain lacking *rpoN*. Moreover, reduced expression of *anr* in
 176 PAO1 Δ *rpoN* was paralleled by an impaired anoxic growth when compared with the wt strain (Fig
 177 2B).

178

179 **Fig 2. RpoN is a major regulator of the *anr* promoter during anaerobiosis.** (A) The strains
 180 PAO1(pME6016anr2) and PAO1 Δ *rpoN*(pME6016anr2) were grown anaerobically in BSM medium
 181 supplemented with 20 mM succinate, 0.2% glutamine and 100 mM KNO₃ (BSM_{anox}). Samples were
 182 withdrawn after 22 hours of static anoxic growth. The bars depict β -galactosidase values resulting
 183 from the activity of the *anr* promoter in PAO1(pME6016anr2) (blue bar) and
 184 PAO1 Δ *rpoN*(pME6016anr2) (orange bar), respectively. Error bars, mean $\pm$ s.d. from four biological
 185 replicates. Statistical analysis was performed using the unpaired two-tailed student *t*-test
 186 (<https://www.graphpad.com>). *****p* < 0.0001 (B) The strains PAO1 (blue squares) and
 187 PAO1 Δ *rpoN* (orange triangles) were grown anaerobically in microtiter plates containing BSM_{anox}
 188 medium. Cell growth was monitored for 24 hours by measuring the absorbance at 600 nm (A₆₀₀)
 189 with a Synergy H1 microplate reader (BioTek). Error bars, mean $\pm$ s.d. of 2 independent
 190 experiments, n = 8 biological replicates, 2 technical replicates.

191

192 As apparent from Fig. 2A, there was considerable *anr* promoter activity in the absence of
 193 RpoN. In addition to RpoN, the sigma factor RpoS and the motility sigma factor FliA were found to
 194 be bound to the *anr* promoter region of strain PA14 [38]. Therefore, we next tested whether the

195 residual transcription of *anr* in the absence of RpoN is RpoS or FliA dependent. First, the *anr*
196 promoter activity was monitored in PAO1 and an isogenic *rpoS* deficient strain. No significant
197 difference in the β -galactosidase activity obtained with the transcriptional *anr-lacZ* fusion gene
198 encoded by pME6016anr2 was observed between the strains (S1 A Fig). Second, the *anr-lacZ* gene
199 expression levels were assessed in strains PAO1 and PAO1 Δ *fliA*, respectively. The lack of FliA
200 resulted in ~1.4-fold decrease in the *anr* promoter activity (S1 A Fig).

201 The screen for regulatory promoter elements revealed as well a putative signature for RpoH
202 (Fig 1, in yellow ochre), overlapping with the RpoN interaction site. To test whether RpoH is also
203 contributing to *anr* transcription, we next monitored the β -galactosidase activity obtained with the
204 transcriptional *anr-lacZ* fusion gene encoded by plasmid pHRP309anr (S1 Table and S1 Text) in
205 the wt and the *rpoH* deficient strain, respectively. As shown in S1 B Fig, the *anr* promoter activity
206 was ~1.5-fold reduced in the absence of RpoH. Interestingly, *rpoH* expression seems to be
207 regulated by the sigma factor AlgU [38,54]. FliA was found to co-immunoprecipitate with the *algU*
208 promoter [38]. Therefore, the detected decrease in the transcription of *anr* in strain PAO1 Δ *fliA* (S1
209 A Fig) might be attributed to indirect effects on RpoH. Taken together, these data indicated a major
210 role of RpoN in regulating the *anr* promoter during anaerobiosis as well as a complex network
211 governing RpoN-independent anoxic *anr* transcription.

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218 **Mutations in the RpoN binding motif and the requirement of the** 219 **transcriptional activator NtrC support direct regulation of the *anr*** 220 **promoter by RpoN**

221

222 To further verify the RpoN dependence of the *anr* promoter, mutations were introduced in the
223 RpoN binding motif upstream of the *anr-lacZ* gene in plasmid pME6016anr2 (Fig. 3, bottom). In

224 plasmids pME6016anr6-8, the -12 box (anr8), the -24 box (anr7) and the -24 and -12 boxes (anr6),
225 were altered, respectively (Fig. 1 and 3). All three mutations resulted in a reduced activity of the *anr*
226 promoter under anaerobic conditions in BSM_{anox} medium. Mutations in the -24 box were similarly
227 detrimental as the alteration of both, the -24 and -12 boxes (Fig 3). These findings corroborated
228 previous ChIP-seq results, showing that RpoN binds to the *anr* promoter region in strain PA14 [38],
229 and are in line with a recent report from Lloyd *et al.*, who observed a 2-fold reduction in *anr*
230 expression upon blocking the RpoN -24 promoter element in strain PAO1 [55].

231

232 **Fig 3. Mutations in the RpoN binding motif reduce *anr* transcription.** The PAO1 cultures were
233 grown anaerobically in BSM_{anox} medium for 22 hours. The bars represent β -galactosidase activities
234 resulting from the *anr* promoter activity obtained with the *anr-lacZ* reporter gene encoded by
235 plasmid pME6016anr2 and variants thereof bearing mutations in the RpoN binding motif. The
236 introduced mutations in the RpoN motif are shown below (letters in blue). Error bars, mean $\pm$ s.d.
237 from three biological replicates. Statistical analysis was performed using the one-way ANOVA
238 followed by Dunnett's multiple comparison test (<https://www.graphpad.com>). **** $p < 0.0001$.

239

240 As mentioned above and depicted in Fig 1, the inspection of the *anr* promoter region
241 revealed the presence of a putative binding site for the transcriptional activator NtrC, which can
242 assist in recruiting the RNA polymerase to RpoN-dependent promoters [56,57]. To determine
243 whether NtrC impacts on the *anr* promoter activity, we assessed the β -galactosidase activities
244 obtained with the transcriptional *anr-lacZ* fusion gene encoded by plasmid pME6016anr2 in the
245 PAO1 wt strain and in a *ntrC* deficient strain. As shown in Fig 4, the absence of NtrC caused a ~3-
246 fold reduction in the *anr* promoter activity upon growth under anaerobic conditions in BSM_{anox}
247 medium.

248

249 **Fig 4. Requirement of NtrC for *anr* transcription under anoxic conditions.** The PAO1 and the
250 PAO1 Δ *ntrC* strains were grown anaerobically in BSM_{anox} medium for 22 hours. The blue and
251 striped bar, respectively, depict the β -galactosidase activities obtained with the transcriptional *anr*-
252 *lacZ* fusion gene encoded by plasmid pME6016anr2 (wt promoter) and plasmid pME6016anr5
253 (deletion of the NtrC motif) in strain PAO1. The green bar depicts the β -galactosidase activity
254 obtained with the transcriptional *anr-lacZ* fusion gene encoded by plasmid pME6016anr2 (wt
255 promoter) in strain PAO1 Δ *ntrC*. Error bars, mean $\pm$ s.d. from three biological replicates. Statistical
256 analysis was performed using the one-way ANOVA followed by Dunnett's multiple comparison
257 test (<https://www.graphpad.com>). **** $p < 0.0001$.

258

259 In addition, the putative NtrC-binding site (nucleotides -124 to -108 relative to the
260 transcriptional start site of *anr*) was deleted in plasmid pME6016anr2, resulting in plasmid
261 pME6016anr5. The deletion of the putative NtrC binding site reduced the *anr* promoter activity ~3-
262 fold when compared with the authentic *anr* upstream region (Fig 4). Taken together, these
263 experiments showed that NtrC is required for efficient *anr* transcription.

264 NtrC is the part of the two-component system NtrBC that functions as the primary regulator
265 of nitrogen metabolism in *Pseudomonas* species. NtrBC activates functions required for the
266 transport and utilization of various nitrogen sources, and together with the two-component system
267 CbrAB maintains the carbon/nitrogen balance in the cell [58-60]. Thus, by regulating PAO1 *anr*
268 transcription, NtrC appears to link the availability of nitrogen sources, such as nitrate or arginine, to
269 anaerobic respiration in PAO1, *i.e.* instead of them being used as a nitrogen source they are
270 preferentially utilized for energy production.

271

272 **RpoN impacts on Anr-dependent and independent functions during**
273 **anaerobiosis**

274 Anr was shown to be required for anaerobic growth and anoxic biofilm formation [1,4,23]. Given
275 that RpoN contributed to a large extent to *anr* transcription during anaerobiosis, we next asked
276 whether *rpoN* is impacting on anoxic biofilm development. The viability and biomass of cultures of
277 PAO1, PAO1 Δ *anr* and PAO1 Δ *rpoN* anoxically grown for 48 hours in BSM_{anox} medium was
278 assessed by live/death staining and visualized by confocal laser scanning microscopy (S2 A Fig).
279 As anticipated, the PAO1 Δ *anr* strain was severely impaired in anoxic biofilm formation. When
280 compared with PAO1, the PAO1 Δ *anr* strain yielded a reduced overall biomass with a high
281 proportion of dead cells (S2 A and B Fig) which is in agreement with the previously described
282 pivotal role of Anr in anaerobic metabolism [19,61]. Although the biomass of anoxic biofilms was
283 likewise reduced in the PAO1 Δ *rpoN* strain, the cells were alive (S2 Fig A and B). On the one hand
284 this observation may be reconciled with a significant decrease but enduring *anr* transcription in the
285 *rpoN* mutant strain (Fig 2A). On the other hand RpoN might impact on Anr-independent functions
286 required for biofilm formation or anaerobic growth [62], which would likewise explain the reduced
287 anaerobic growth (Fig. 2B) and the diminished anoxic biofilm formation seen with the PAO1 Δ *rpoN*
288 strain (S2 A and B Fig).

289 We therefore next asked whether complementation the *anr* gene from plasmid pMMB-*anr*
290 would rescue the anoxic growth defect observed with strain PAO1 Δ *rpoN* (Fig. 2B; S2 A and B
291 Fig). As anticipated, in contrast to strain PAO1 Δ *anr*(pMMB Δ *rbs*) harbouring the parental vector,
292 ectopic expression of *anr* in strain PAO1 Δ *anr*(pMMB-*anr*) rescued the growth defect (S2 C Fig).
293 However, ectopic expression of *anr* in strain PAO1 Δ *rpoN*(pMMB-*anr*) did not complement the
294 Δ *rpoN* growth phenotype (S2 C Fig), again indicating that RpoN might impact on Anr-independent
295 functions or even indirectly affect the activity of Anr. For instance, previous transcriptome studies
296 suggested that genes involved in formation of [4Fe-4S]²⁺ clusters, which are required for activity of
297 Anr, were down-regulated in a *rpoN* deficient mutant [38].

298 Next, we were seeking for an assay to test whether the activity of Anr is impaired in the
299 absence of RpoN. We have previously shown that transcription from the promoter of the sRNA

300 PhrS depends strictly on functional Anr [22]. Therefore, a transcriptional *phrS-lacZ* reporter gene
301 encoded by plasmid pME7233 [22] was employed and the β -galactosidase activity was monitored
302 in strains PAO1, PAO1 Δ *anr* and PAO1 Δ *rpoN* harbouring additionally plasmid pMMB-*anr* and the
303 control plasmid pMMB Δ *rbs*, respectively. As anticipated, the *phrS* promoter was inactive in the *anr*
304 deficient strain, and ectopic expression of *anr* restored *phrS* transcription above levels observed
305 with the PAO1 wt strain (Fig. 5). When compared with the PAO1 wt strain, transcription of *phrS*
306 was reduced in the *rpoN* deficient strain (Fig. 5 (bar 3), lower panel) , which concurred with
307 reduced *anr* transcript levels (Fig 5, top panel). However, upon ectopic expression of *anr* in the
308 *rpoN* mutant strain, the *phrS* transcript levels (Fig. 5, top panel) and promoter activity (Fig. 5 (bar
309 4), lower panel) were restored to similar levels as in the wt strain. As the activity of Anr was
310 apparently not significantly affected in strain PA14 Δ *rpoN*(pME7233; pMMB-*anr*), the observed
311 impairment in anoxic growth (Fig. 2B) and biofilm formation in the absence RpoN (S2 Fig), can
312 thus most likely be reconciled with the findings that RpoN controls other factors related to biofilm
313 formation like pili and flagellar formation [30,31] or quorum sensing [33,35,64]. Nevertheless,
314 RpoN also contributes to Anr-mediated regulation under anoxic conditions as full-fledged
315 transcription of *anr* required RpoN (Fig. 2A and 5 (bar 4), top panel).

316 A subset of genes within the RpoN regulon is known to be Anr regulated [38,54,63]. Here,
317 we have shown (Fig 5) that RpoN indirectly affects Anr-dependent *phrS* transcription, encoding for
318 a base-pairing sRNA involved in linking oxygen sensing to quorum sensing [22]. Moreover, by
319 blocking the RpoN binding sites in PAO1wt, the expression of Anr-dependent genes essential for
320 arginine metabolism, redox reactions, TCA cycle and LPS formation was also diminished [54],
321 corroborating that RpoN indirectly regulates these genes through its impact on *anr* transcription.

322 An example of RpoN and Anr dual regulation of a target involved in biofilm formation
323 represents the regulatory RNA CrcZ [64,65]. CrcZ requires RpoN and the two component system
324 CbrAB for transcription [66]. In addition, CrcZ levels are strongly increased in anoxic biofilms
325 when compared with planktonically growing cells, which balances biofilm formation in strain PA14

[67]. A putative Anr binding site was identified in the *crcZ* promoter region and *crcZ* was poorly expressed in the absence of Anr [67]. Thus, during anaerobiosis RpoN seems to regulate the *CrcZ* RNA by directly promoting its transcription, and indirectly by contributing to the expression of *anr*.

Fig 5. Ectopic expression of *anr* restores *phrS* promoter activity in the absence of RpoN. The cultures were grown anaerobically in BSM_{anox} medium for 22 hours. The bars represent the β -galactosidase values obtained with the transcriptional *phrS-lacZ* reporter gene encoded by plasmid pME7233 in strains PAO1(pME7233; pMMB Δ *rbs*) (bar 1), PAO1(pME7233; pMMB-*anr*) (bar 2), PAO1 Δ *rpoN*(pME7233; pMMB Δ *rbs*) (bar 3), PAO1 Δ *rpoN*(pME7233; pMMB-*anr*) (bar 4), PAO1 Δ *anr*(pME7233; pMMB Δ *rbs*) (bar 5) and PAO1 Δ *anr*(pME7233; pMMB-*anr*) (bar 6). Error bars, mean $\pm$ s.d. from two independent experiments. Statistical analysis was performed using the one-way ANOVA followed by Tukey's post-hoc test (<https://www.graphpad.com>). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The *anr* mRNA levels (top panels) were determined by RT-PCR. The *rpoD* mRNA served as a loading control.

In summary, our data indicate that the expression of the anaerobic regulator Anr is modulated by several sigma factors. While RpoN in concert with NtrC is required for full-fledged *anr* transcription in PAO1, RpoH and FliA are directly or indirectly adding to the *anr* promoter activity during anaerobiosis. Cystic fibrosis airways are characterized by steep oxygen gradients [68], in which the invasion of neutrophils contributes to oxygen deprivation and results in formation of reactive oxygen species [69]. Thus, it is not surprising that sigma factors known to be involved in regulation of adaptation and protection (FliA and RpoH) [38,70,71], chemotaxis (FliA and RpoN) [36,38], motility and attachment (FliA, RpoN and RpoH) [30,31,36,38,44,45] are affecting *anr* expression. A better understanding of the regulatory events governing anoxic growth and biofilm formation of *P. aeruginosa* in chronic infections may aid in developing novel therapeutic strategies.

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355

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561 **Supporting information**

562 **S1 Text. Supporting materials and methods.**

563 **S1 Table. Strains and plasmids used in this study.**

564 **S2 Table. DNA oligonucleotides used in this study.**

565 **S1 Fig. Regulation by FliA, RpoS and RpoH of *anr* transcription during anaerobiosis.** (A) The
566 strains PAO1, PAO1 Δ *fliA* and PAO1 Δ *rpoS* harbouring plasmid pME6016anr2, respectively, were
567 grown anaerobically in BSM_{anox} medium. Samples were taken after 22 hours of anoxic static
568 growth. The bars depict β -galactosidase obtained with the *anr-lacZ* reporter gene in PAO1 (blue),
569 PAO1 Δ *fliA* (red) and PAO1 Δ *rpoS* (violet), respectively. Error bars, mean $\pm$ s.d. from five
570 biological replicates. Statistical analysis was performed using the one-way ANOVA followed by
571 Dunnett's multiple comparison test (<https://www.graphpad.com>). ns (not significant), * $p < 0.05$. (B)
572 The strains PAO1 and PAO1 Δ *rpoH* harbouring pHRP309anr, respectively, were grown
573 anaerobically in BSM medium supplemented with 20 mM succinate, 0.2% glutamine and 100 mM
574 KNO₃ (BSM_{anox} medium). Samples were taken after 22 hours of static anoxic growth. The bars
575 depict β -galactosidase values obtained with the *anr-lacZ* reporter gene in PAO1 (blue) and
576 PAO1 Δ *rpoH* (yellow), respectively. Error bars, mean $\pm$ s.d. from four biological replicates.
577 Statistical analysis was performed using the unpaired two-tailed student *t*-test
578 (<https://www.graphpad.com>). ** $p < 0.01$.

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580 **S2 Fig. Anoxic biofilm formation of strains PAO1, PAO1 Δ *anr* and PAO1 Δ *rpoN*** (A) The
581 distribution of live and dead cells in static anoxic biofilms of PAO1, PAO1 Δ *anr* and PAO1 Δ *rpoN*
582 strain, respectively, was visualized by confocal laser scanning microscopy. Live cells (green) are
583 stained with Syto 9 fluorescent dye and dead cells (red) are visualized with propidium iodide.
584 Three-dimensional images (x, y, z-axes) of biofilms are shown (scale-bars represent 10 μ m). (B)
585 The biomass of live (green) and dead (red) cells in static anoxic biofilms of PAO1, PAO1 Δ *anr* and

586 PAO1 $\Delta rpoN$, respectively, were quantified from confocal laser scanning microscopy vertical image
587 stacks using Comstat2 software [53]. Error bars, mean $\pm$ s.d. from three biological replicates.
588 Statistical analysis was performed using the one-way ANOVA followed by Dunnett's multiple
589 comparison test. ****P** < 0.01. (C) The strains PAO1 $\Delta rpoN$ (pMMB Δrbs) (orange circles),
590 PAO1 $\Delta rpoN$ (pMMB-*anr*) (orange squares), PAO1 Δanr (pMMB Δrbs) (grey circles) and
591 PAO1 Δanr (pMMB-*anr*) (grey squares) were grown anaerobically in microtiter plates containing
592 BSM_{anox} medium. Cell growth was monitored for 24 hours by measuring absorbance at 600 nm
593 (A_{600}) with a Synergy H1 microplate reader (BioTek). Error bars, mean $\pm$ s.d. of 2 independent
594 experiments, n= 2 biological replicates, 2 technical replicates.

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Figure 1

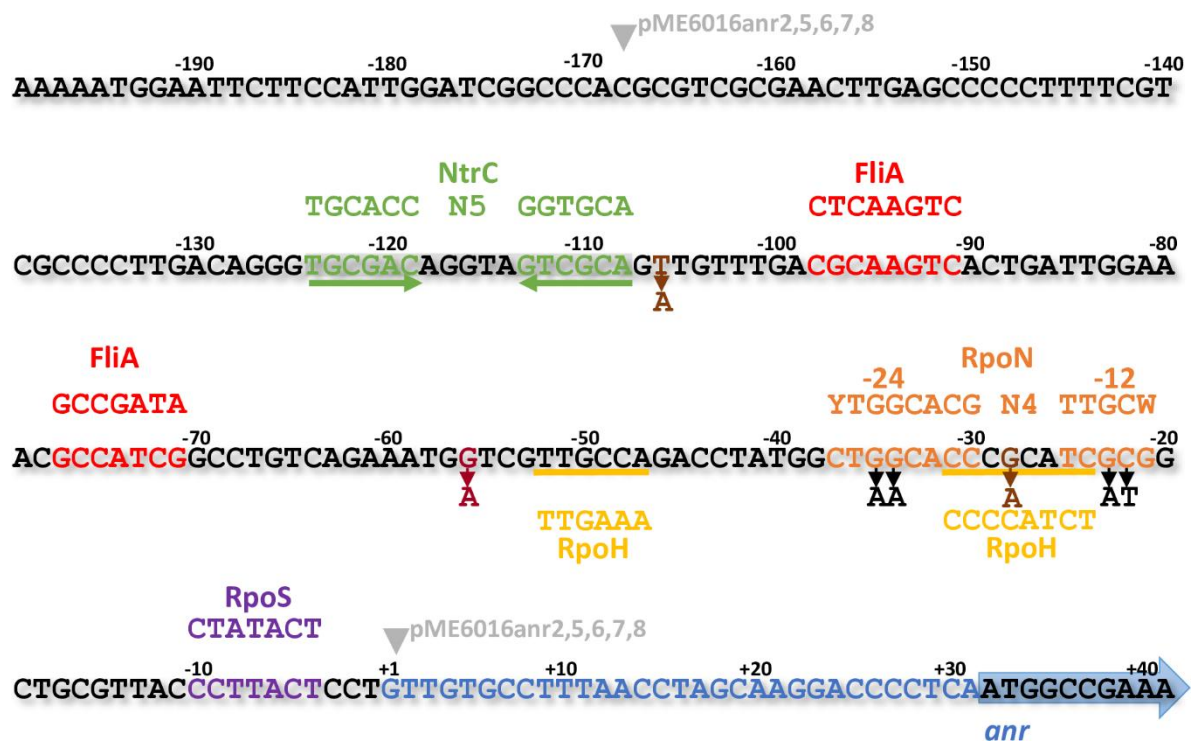


Figure 2

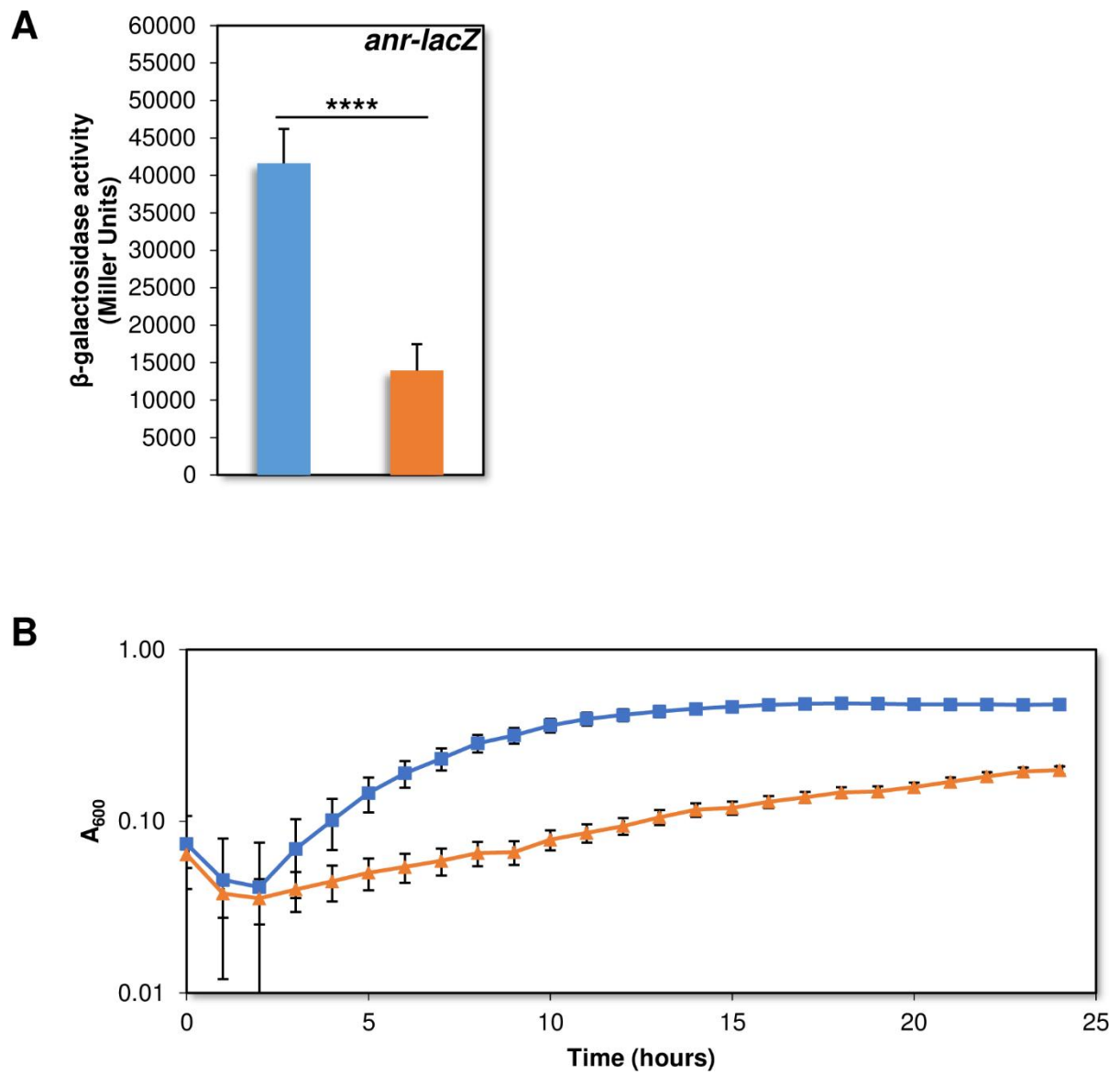
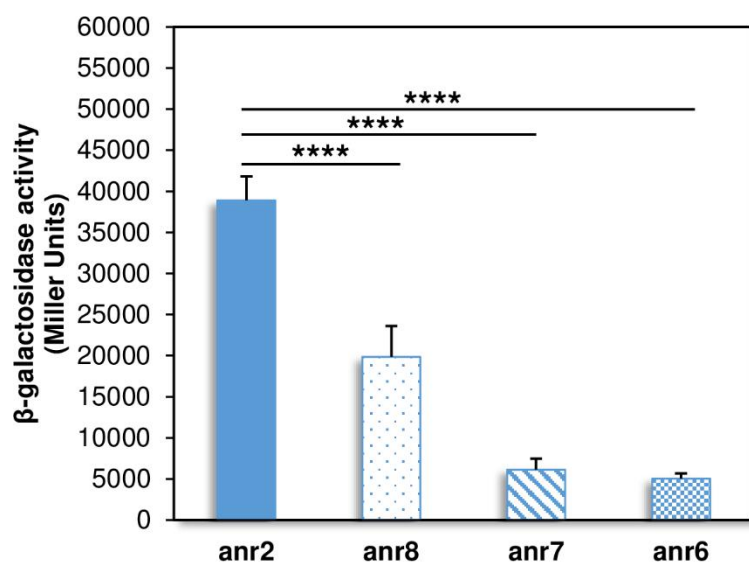


Figure 3



	RpoN consensus	
	-24	-12
	CTGGCACC CGCATCGCG	
anr2 (wt)	GG	CG
anr8	GG	AT
anr7	AA	CG
anr6	AA	AT

Figure 4

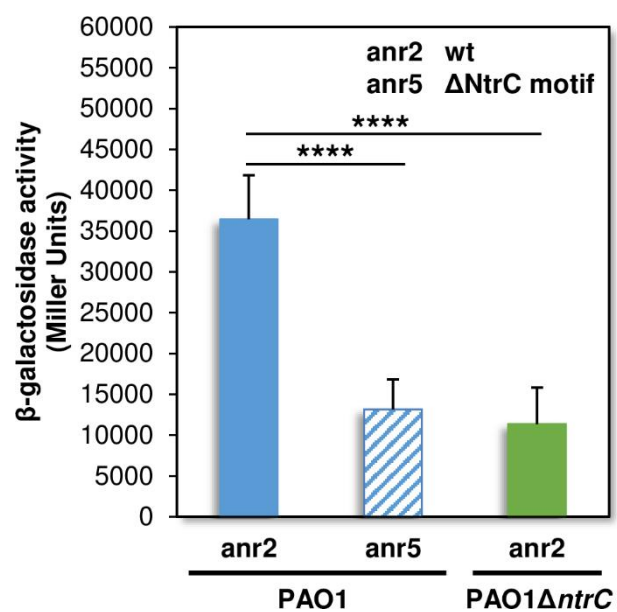
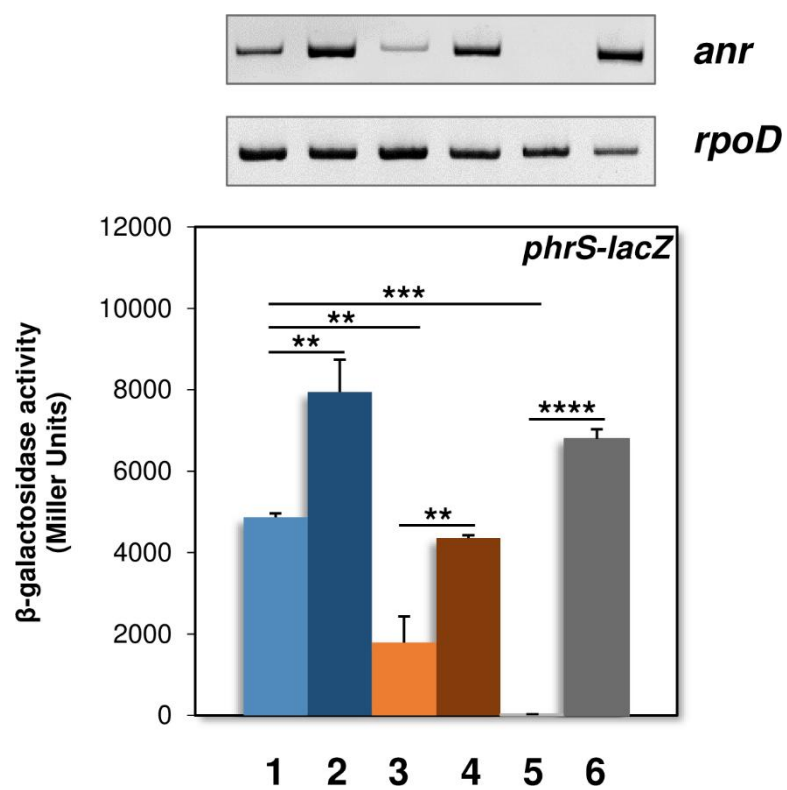


Figure 5



5.2.1. Supplementary Figures and Tables

Supporting information

RpoN is required for full-fledged anoxic transcription of the anaerobic regulator gene *anr* in *Pseudomonas aeruginosa* O1

Petra Pusic, Udo Bläsi* and Elisabeth Sonnleitner*

Department of Microbiology, Immunobiology and Genetics, Center of Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter, Dr. Bohrgasse 9/4, 1030 Vienna, Austria

*Corresponding author

Elisabeth Sonnleitner

E-mail: elisabeth.sonnleitner@univie.ac.at

Udo Bläsi

E-mail: udo.blaesi@univie.ac.at

S1 Text

Construction of plasmids used in this study

The genome coordinates are given according to the *Pseudomonas* genome database (for PAO1) [71].

Plasmid pME6016anr2: The fragment (genome coordinates: 1682004-1681836) including nucleotides -168 to +1 relative to the *anr* transcriptional start site was amplified by PCR using the oligonucleotides Y83 and Z83 (S2 Table) and chromosomal DNA of PAO1 as template. The PCR fragment was cleaved with EcoRI and PstI and then ligated into the corresponding sites of plasmid pME6016.

Plasmid pME6016anr5 encodes a transcriptional *anr-lacZ* fusion gene, wherein the NtrC motif in the *anr* promoter was deleted. First, two PCR products flanking the NtrC binding site of *anr* (genome coordinates: 1682004-1681961 and 1681943-1681836, respectively) were obtained from PAO1 chromosomal DNA with the primer pairs Y83/L94 and M94/Z83 (S2 Table), respectively. The combined PCR fragments including nucleotides -168 to -125 and -107 to +1, respectively, relative to the *anr* transcriptional start were used as a template for a second overlapping PCR with primers Y83 and Z83. The resulting fragment was cleaved with EcoRI and PstI and ligated into the corresponding sites of pME6016.

Plasmid pME6016anr6 encodes a transcriptional *anr-lacZ* fusion gene, wherein the RpoN motif was altered to GG → AA in the -24 box and GC → AT in the -12 box. The fragment (genome coordinates: 1682004-1681836) including nucleotides -168 to +1 relative to the *anr* transcriptional start site was amplified by PCR using chromosomal DNA of PAO1 as template together with the oligonucleotides Y83 and X98 (S2 Table), the latter of which introduced the corresponding

mutations. The PCR fragment was cleaved with EcoRI and PstI and ligated into the corresponding sites of plasmid pME6016.

Plasmid pME6016anr7 encodes a transcriptional *anr-lacZ* fusion gene wherein the RpoN motif was altered to GG → AA in the -24 box. The fragment (genome coordinates: 1682004-1681836) including nucleotides -168 to +1 relative to the *anr* transcriptional start site was amplified by PCR using chromosomal DNA of PAO1 as template together with the oligonucleotides Y83 and V98 (S2 Table), the latter of which introduced the corresponding mutations. The PCR fragment was cleaved with EcoRI and PstI and ligated into the corresponding sites of plasmid pME6016.

Plasmid pME6016anr8 encodes a transcriptional *anr-lacZ* fusion gene wherein the RpoN motif was altered to GC → AT in the -12 box. The fragment (genome coordinates: 1682004-1681836) including nucleotides -168 to +1 relative to the *anr* transcriptional start site was amplified by PCR using chromosomal DNA of PAO1 as template and the oligonucleotides Y83 and W98 (S2 Table), the latter of which introduced the mutations. The PCR fragment was cleaved with EcoRI and PstI and ligated into the corresponding sites of plasmid pME6016.

Plasmid pHRP309anr: The fragment (genome coordinates: 1682341-1681836) including nucleotide -505 to +1 relative to *anr* transcription start site was amplified by PCR using chromosomal DNA of PAO1 as template and the oligonucleotides M90 and N90 (S2 Table). The PCR fragment was cleaved with XbaI and KpnI and then ligated into to the corresponding sites of plasmid pHRP309.

The insertion of the respective fragments into the different plasmids was verified by DNA sequencing.

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Table S1. Strains and plasmids used in this study.

Strains/plasmids		Genotype or relevant features ^a	Reference or source
<i>P. aeruginosa</i>			
PAO1	wild-type		[72]
PAO1 Δ <i>anr</i>	PAO6261		[8]
PAO1 Δ <i>rpoN</i>	PAO6358		[62]
PAO1 Δ <i>ntrC</i>	PAO6743; <i>ntrC::aacCI</i> ; Gm ^R		K. Lapouge, Lausanne
PAO1 Δ <i>rpoH</i>	PAO376(<i>rpoH</i>)::IS <i>phoA/hah</i> ; Tet ^R		[73]
PAO1 Δ <i>fliA</i>	<i>fliA</i> deletion mutant		[45]
PAO1 Δ <i>rpoS</i>	PAO1 <i>rpoS101::aacCI</i> ; Gm ^R		[46]
<i>E. coli</i>			
DH5 α	<i>recA1 endA1 hsdR17 thi-1 supE44 gyrA96 relA1 deoR</i> <i>Δ(lacZYA-argF) U169 (Φ80lacZAM15)</i>		[74]
Plasmids			
pME6016	Cloning vector for transcriptional <i>lacZ</i> fusions; Tc ^R		[75]
pME6016anr2	pME6016 with transcriptional <i>anr</i> (-168 to +1)- <i>lacZ</i> fusion		This study
pME6016anr5	pME6016anr2 with a deletion of the NtrC binding site (nucleotides -124 to -108)		This study
pME6016anr6	pME6016anr2 with a GG→AA mutation in the -24 box and a GC→AT mutation in the -12 box of the RpoN-dependent promoter		This study
pME6016anr7	pME6016anr2 with a GG→AA mutation in the -24 box of the RpoN-dependent promoter		This study
pME6016anr8	pME6016anr2 with a GC→AT mutation in the -12 box of the RpoN-dependent promoter		This study
pHRP309	Cloning vector for transcriptional <i>lacZ</i> fusions; Gm ^R		[76]
pHRP309anr	pHRP309 with transcriptional <i>anr</i> (-505 to +1)- <i>lacZ</i> fusion		This study
pMMB Δ <i>rbs</i>	pMMB67HE with a deletion of the RBS; Cb ^R		[77]
pMMB- <i>anr</i>	pMMB Δ <i>rbs</i> carrying <i>anr</i> under transcriptional control of Ptac		[78]
pME7233	Transcriptional <i>phrS-lacZ</i> fusion; Tc ^R		[22]

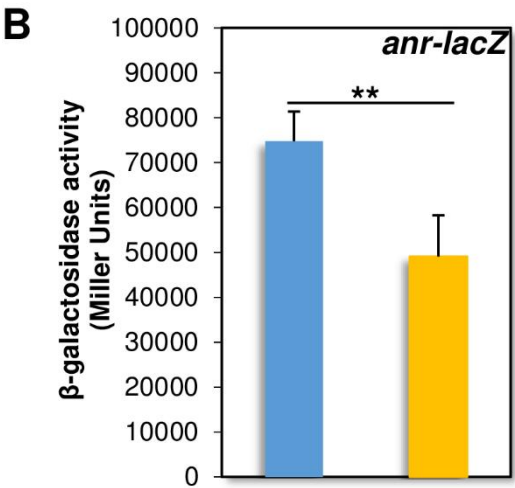
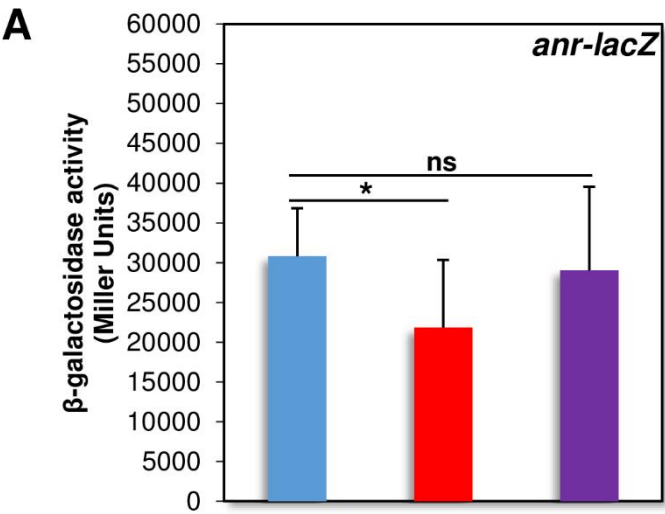
^a The coordinates are given with respect to the *anr* transcriptional start site.

Table S2. DNA oligonucleotides used in this study.

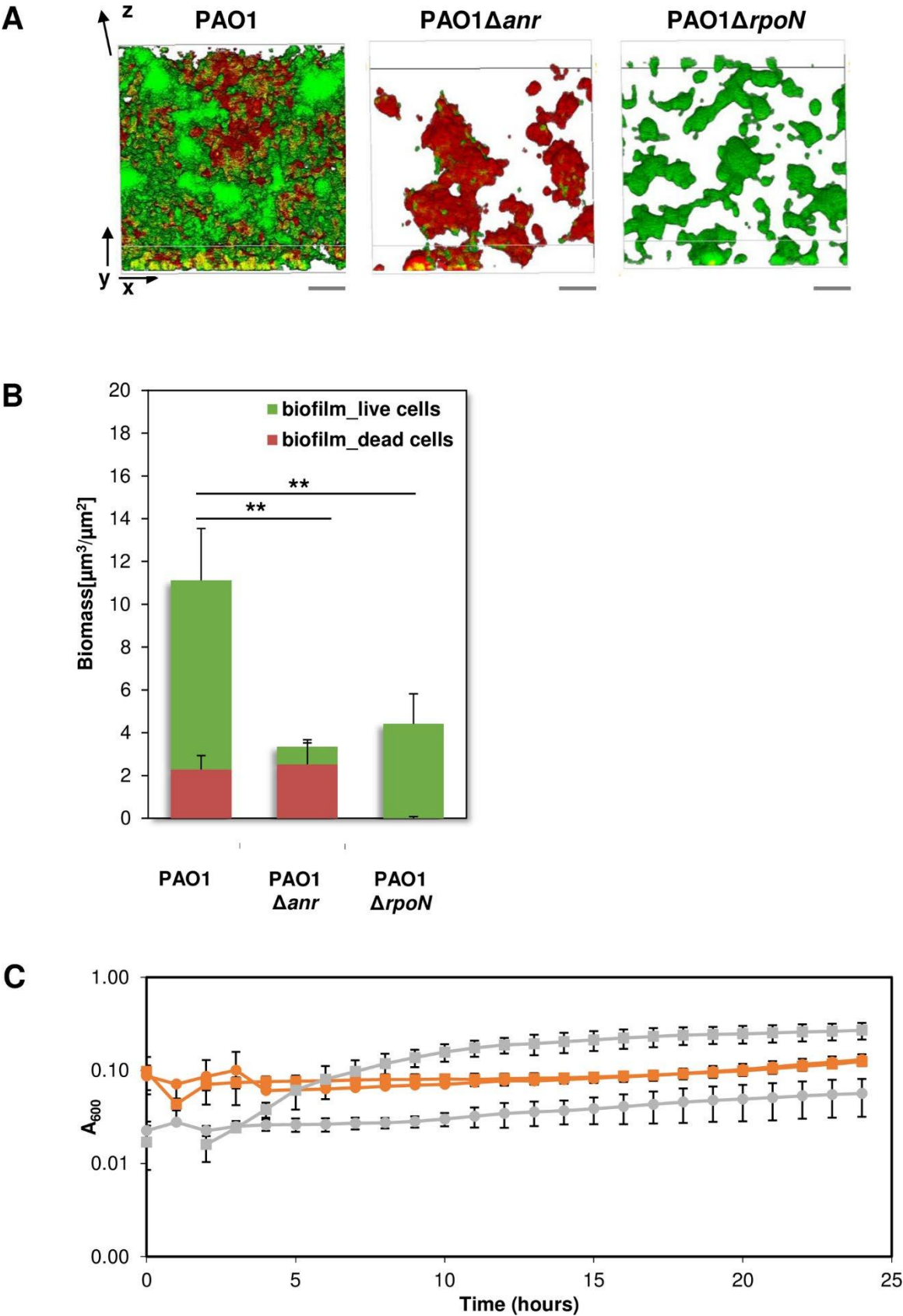
Oligo	Sequence ^a	Restriction site
L94	CGTCAAACAACCCCTGTCAAGGGGCGAC	
M94	CCTTGACAGGGGTTGTTTGACGCAAGTCACTG	
V98	AAA <u>ACTGCAG</u> CAGGAGTAAGGGTAACGCAGCCGCGATGCGGGTGT <u>TT</u> AGCCATAGG	PstI
W98	AAA <u>ACTGCAG</u> CAGGAGTAAGGGTAACGCAGCC <u>AT</u> GATGCGGGTGCCAGCCATAGG	PstI
X98	AAA <u>ACTGCAG</u> CAGGAGTAAGGGTAACGCAGCC <u>AT</u> GATGCGGGTGT <u>TT</u> AGCCATAGG	PstI
Y83	GCTAG <u>GAATT</u> CCGCGTCGCGAACTTGAG	EcoRI
Z83	GATC <u>CTGCAG</u> CAGGAGTAAGGGTAACGCAGC	PstI
M90	TCGAT <u>CTAG</u> ACTGCAGCTCCCTGAGCTTCT	XbaI
N90	GATC <u>GGTAC</u> CCAGGAGTAAGGGTAACGCAGC	KpnI
I129	CACCTTCCTGGTCAACCTGT	
J129	CTCGATGGAGTCGAGGATGT	
Q117	AAGGCCCTGAAGAAGCACGG	
R117	GATCGGCATGAACAGCTCGG	

^a Restriction sites are underlined; for oligos X98, V98 and W98, the mutations introduced in to the *anr* sequence are highlighted in bold and underlined.

Supplementary Figure S1



Supplementary Figure S2



5.3 Harnessing metabolic regulation to increase Hfq-dependent antibiotic susceptibility in *Pseudomonas aeruginosa*



Harnessing Metabolic Regulation to Increase Hfq-Dependent Antibiotic Susceptibility in *Pseudomonas aeruginosa*

Petra Pusic^{1†}, Elisabeth Sonnleitner^{1*†}, Beatrice Krennmayr¹, Dorothea A. Heitzinger¹, Michael T. Wolfinger², Armin Resch¹ and Udo Bläsi^{1*}

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United Kingdom

*Correspondence:

Elisabeth Sonnleitner
elisabeth.sonnleitner@univie.ac.at
Udo Bläsi
udo.blaesi@univie.ac.at

[†]These authors have contributed
equally to this work

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¹ Max F. Perutz Laboratories, Department of Microbiology, Immunobiology and Genetics, Vienna Biocenter, University of Vienna, Vienna, Austria, ² Department of Theoretical Chemistry, University of Vienna, Vienna, Austria

The opportunistic human pathogen *Pseudomonas aeruginosa* is responsible for ~ 10% of hospital-acquired infections worldwide. It is notorious for its high level resistance toward many antibiotics, and the number of multi-drug resistant clinical isolates is steadily increasing. A better understanding of the molecular mechanisms underlying drug resistance is crucial for the development of novel antimicrobials and alternative strategies such as enhanced sensitization of bacteria to antibiotics in use. In *P. aeruginosa* several uptake channels for amino-acids and carbon sources can serve simultaneously as entry ports for antibiotics. The respective genes are often controlled by carbon catabolite repression (CCR). We have recently shown that Hfq in concert with Crc acts as a translational repressor during CCR. This function is counteracted by the regulatory RNA CrcZ, which functions as a decoy to abrogate Hfq-mediated translational repression of catabolic genes. Here, we report an increased susceptibility of *P. aeruginosa* hfq deletion strains to different classes of antibiotics. Transcriptome analyses indicated that Hfq impacts on different mechanisms known to be involved in antibiotic susceptibility, viz import and efflux, energy metabolism, cell wall and LPS composition as well as on the c-di-GMP levels. Furthermore, we show that sequestration of Hfq by CrcZ, which was over-produced or induced by non-preferred carbon-sources, enhances the sensitivity toward antibiotics. Thus, controlled synthesis of CrcZ could provide a means to (re)sensitize *P. aeruginosa* to different classes of antibiotics.

Keywords: *Pseudomonas aeruginosa*, Hfq, antibiotic susceptibility, c-di-GMP, membrane potential

INTRODUCTION

P. aeruginosa is a Gram-negative opportunistic pathogen that is associated with a broad spectrum of acute and chronic infections (Kerr and Snelling, 2009). *P. aeruginosa* can be devastating for immunocompromised individuals or patients with cystic fibrosis, leading to high morbidity and mortality (Williams et al., 2010). A major burden to eradicate *P. aeruginosa* infections is its high intrinsic resistance against several classes of antibiotics (Poole, 2011) as well as its ability to form biofilms. Biofilms are recalcitrant to antibiotic treatment due to restricted penetration and their altered physiology (Ciofu et al., 2015). One of the key signals for the transition from mobile to sessile

lifestyle is the signaling molecule cyclic-3',5'-diguanylic acid (c-di-GMP) (Valentini and Filloux, 2016). High c-di-GMP levels correlate with biofilm formation and low c-di-GMP levels with planktonic growth (Simm et al., 2004; Basu Roy and Sauer, 2014). The cellular content of c-di-GMP is established by the interplay between diguanylate cyclases (DGC) and phosphodiesterases (PDE) that are responsible for the synthesis and degradation of c-di-GMP, respectively (Valentini and Filloux, 2016). The c-di-GMP levels are sensed by c-di-GMP binding receptors/effectors (Valentini and Filloux, 2016; Moradali et al., 2017), which then activate exopolysaccharide production (Psl, Pel, alginate), cell aggregation and biofilm formation, and interfere with the ATPase activity of the master regulator of flagellar biosynthesis FleQ (Su et al., 2015). Moreover, elevated levels of c-di-GMP can interfere with antibiotic resistance in a manner unrelated to biofilm growth (Hoffman et al., 2005; Gupta et al., 2013, 2014; Nicastro et al., 2014). In addition to the c-di-GMP signaling pathway, the Gac/Rsm network also regulates the switch between planktonic and biofilm lifestyle (Mikkelsen et al., 2011). The Rsm system in *P. aeruginosa* consists of two RNA binding proteins (RsmA and RsmN) and four regulatory RNAs (RsmY, RsmZ, RsmW, RsmV) that function by sequestering RsmA and RsmN from mRNA targets (Pessi et al., 2001; Heurlier et al., 2004; Kay et al., 2006; Marden et al., 2013; Morris et al., 2013; Miller et al., 2016; Janssen et al., 2018). The regulatory RNAs are transcribed under different growth conditions and at least two of them, RsmZ and RsmY, are under the control of the two component system GacA/S (Kay et al., 2006; Miller et al., 2016; Janssen et al., 2018). Moreover, the levels of RsmZ are dependent on RNase G (CafA), the synthesis of which is controlled by the two component system BfrSR in a biofilm specific manner (Petrova and Sauer, 2010). There are some indications that c-di-GMP signaling and Gac/Rsm mediated regulation are interlinked (Frangipani et al., 2014; Moscoso et al., 2014). Most likely, RsmA and RsmN affect the synthesis of DGCs, like SiaD, RoeA and SadC at the post-transcriptional level (Moscoso et al., 2014; Romero et al., 2018).

Sub-populations of *P. aeruginosa* biofilms respond differently to antibiotic treatment due to their altered metabolic activity (Pamp et al., 2008). Similarly, different environmental cues impact on antimicrobial susceptibility (Martínez and Rojo, 2011; Poole, 2012), which has been attributed to regulators such as Crc (Linares et al., 2010), CbrA (Yeung et al., 2011) or PhoPQ (Schurek et al., 2009). Moreover, small regulatory RNAs (sRNA), which commonly act in response to stressors, can act as post-transcriptional regulators of genes involved in antibiotic susceptibility (Dersch et al., 2017). For example, the *P. aeruginosa* sRNAs Sr0161 and ErsA control meropenem resistance by binding to the 5' UTR of *oprD* (Zhang et al., 2017), encoding an uptake channel for carbapenems. The *P. aeruginosa* sRNA Sr006 activates the expression of *pagL*, encoding an enzyme responsible for deacylation of lipid A, which results in polymyxin resistance (Zhang et al., 2017).

In *P. aeruginosa*, the global regulator Hfq is involved in sRNA-mediated riboregulation as well as carbon catabolite repression (CCR) (Sonnleitner and Bläsi, 2014; Ferrara et al., 2015; Sonnleitner et al., 2017; Zhang et al., 2017). The function of Hfq in riboregulation in Gram-negative bacteria has been well

established. It stabilizes sRNAs and facilitates their annealing with target mRNAs (Santiago-Frangos and Woodson, 2018). In *P. aeruginosa*, Hfq was shown to bind directly to catabolic mRNAs to block translation during CCR (Sonnleitner and Bläsi, 2014). Hereby, the catabolite repression protein Crc acts as a co-repressor by stabilizing the RNA-Hfq complex (Sonnleitner et al., 2018). The response to different C-sources is mediated through different levels of the regulatory RNA CrcZ (Sonnleitner et al., 2009; Valentini et al., 2014). CrcZ can sequester Hfq when CCR is relieved (Sonnleitner and Bläsi, 2014; Sonnleitner et al., 2018). CrcZ expression is under control of the alternative sigma factor RpoN and the two component system CbrA/B (Sonnleitner et al., 2009; Abdou et al., 2011). The signal responsible for CbrA activation remains unknown, but it is thought to be related to the internal energy status of the cell (Valentini et al., 2014). Furthermore, as CrcZ acts as a decoy for Hfq, CrcZ does not only interfere with direct translational repression by Hfq-Crc (Sonnleitner and Bläsi, 2014) but also with Hfq-mediated riboregulation by sRNAs (Sonnleitner et al., 2017).

There is limited knowledge on the impact of Hfq on antibiotic susceptibility in bacteria (Yamada et al., 2010; Kim et al., 2015). The increased sensitivity of an *E. coli* *hfq* mutant strain toward several toxic compounds coincided with reduced quantities of ArcB, which is a component of the ArcAB-TolC efflux pump. Further studies indicated that Hfq positively regulates *arcB* at the post-transcriptional level (Yamada et al., 2010). As mentioned above, there are some examples in which functions involved in antibiotic susceptibility are directly or indirectly regulated by Hfq-mediated riboregulation via sRNAs (Dersch et al., 2017; Zhang et al., 2017). Here, we studied the susceptibility of *P. aeruginosa* PAO1 and PA14 *hfq* deletion strains toward several classes of clinically relevant antibiotics. In general, the *hfq* deletion resulted in an increased sensitivity toward the antibiotics. A RNA_{Seq} based transcriptome analysis of PA14Δ*hfq* indicated that Hfq impacts on several antibiotic resistance determinants. Moreover, the *hfq* deletion strains were impaired in energy metabolism and have reduced c-di-GMP levels. Furthermore, we show that cross-regulation by the regulatory RNA CrcZ, which titrates Hfq, renders *P. aeruginosa* wild-type strains more susceptible to antibiotics. Induced synthesis of CrcZ in complex synthetic cystic fibrosis medium reduced the minimal inhibitory concentration (MIC) toward gentamicin and cefepime.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions

The *P. aeruginosa* strains PAO1 (Holloway et al., 1979), PAO1Δ*hfq* (Sonnleitner et al., 2017), PA14 (Liberati et al., 2006), PA14Δ*hfq* (Wurtzel et al., 2012), and the plasmids pMMB67HE (vector control; Fürste et al., 1986) and pMMB*crcZ* (*P_{tac}* driven constitutive expression of *crcZ*; Sonnleitner and Bläsi, 2014) have been described. As indicated in the text, the cultures were grown at 37°C in either synthetic cystic fibrosis medium (SCFM) (Palmer et al., 2007) containing 100 μM FeSO₄ (Tata et al.,

2016), Lysogeny-broth (LB) (Sambrook et al., 1989) or Basal-Salt medium (BSM) supplemented with the indicated carbon sources (Sonnleitner et al., 2009).

Construction of Plasmid pTCglpT

To construct a transcriptional gene fusion between *glpT* and *lacZ*, a 468-bp fragment containing the *glpT* promoter region (nt −495 to −27 with regard to the A (+1) of the start codon) was amplified by PCR using the oligonucleotides D121 (5′-TTT TTG AAT TCC CTT CGC TGC CGG CCA ACC-3′) and E121 (5′-TTT TTC TGC AGG CCT TTT TAC GCG GTT GC-3′) and chromosomal DNA of PA14 as template. The PCR fragment was cleaved with *EcoRI* and *PstI*, and then ligated into the corresponding sites of plasmid pME6016 (Schnider-Keel et al., 2000).

Effect of Gentamicin on PA14 and PA14Δhfq Grown to Early Stationary Phase

Overnight cultures of PA14 and PA14Δhfq were inoculated in SCFM at an initial OD₆₀₀ of 0.05, and the cultures were further grown aerobically to an OD₆₀₀ of 1.8–2.0. Then, 180 μl aliquots were transferred to 96-flat-bottom-well microtiter plates (Thermo Scientific) and 20 μl of SCFM containing serial dilutions of gentamicin (0.5–4 mg/L) were added. Static growth was monitored at 37°C for 18 h in a SynergyH1 microplate reader (Biotek). From these initial studies it became obvious that a gentamicin concentration of 2 mg/L resulted in a stronger growth retardation of PA14Δhfq when compared with PA14 (not shown). Therefore, the growth experiment was repeated in the presence of gentamicin at a final concentration of 2 mg/L. Samples were withdrawn at an OD₆₀₀ of 1.8–2.0 (initial inoculum) and after 18 h of incubation in the presence of 2 mg/L gentamicin. The samples were serially diluted and plated on LB agar plates. The plates were incubated overnight aerobically at 37°C to determine the CFU. All experiments were performed in duplicates and the results are presented as mean ± standard deviation.

Determination of the Minimal Inhibitory Concentration (MIC) by Evaluator Strips

Bacterial cultures were grown in the media as indicated in the text to an OD₆₀₀ of 1.8–2.0. Then, either 200 μl of cultures (high inoculum, corresponding to 4×10^9 CFU/mL) or 100 μl of 1/1,000 dilution of the respective cultures (low inoculum, corresponding to 2×10^6 CFU/mL) were plated on agar plates containing the respective media. MIC Evaluator strips were applied with the following antibiotic concentrations: cefepime, 0.016–256 mg/L (bioMérieux); ciprofloxacin, 0.002–32 mg/L (Oxoid); colistin, 0.016–256 mg/L (bioMérieux); fosfomycin, 0.064–1,024 mg/L (bioMérieux); gentamicin, 0.015–256 mg/L (Oxoid); imipenem, 0.002–32 mg/L (Oxoid); tetracycline, 0.015–256 mg/L (Oxoid). The plates were incubated at 37°C and the MICs were determined by analyzing the growth inhibition zones. The MICs correspond to the lowest concentration of antibiotics that impeded growth.

Agar Disc Diffusion

PA14 and PAO1 were grown in BSM supplemented with either 40 mM succinate or oxaloacetate to an OD₆₀₀ of 1.8–2.0. Then 200 μl of cultures were plated on agar plates containing the respective media and filter discs were applied on top with the following antibiotic concentrations: cefepime (Sigma), 60 μg; colistin (Oxoid), 25 μg; ciprofloxacin (Oxoid), 5 μg; gentamicin (Roth), 120 μg. The filter discs for colistin and ciprofloxacin were purchased commercially (Oxoid), the other discs were self-made. The plates were incubated at 37°C. The diameter of the growth inhibition zones was measured and normalized to that obtained on BSM-succinate, which was set to 100%.

MIC Determination by Microdilution in Glass Tubes

PA14, PAO1 and the PA14 and PAO1 strains, harboring either plasmid pMMB67HE or plasmid pMMBcrcZ, were grown in SCFM medium to an OD₆₀₀ of 1.0. Then, 1 ml of culture was mixed with 1 ml of SCFM medium containing serial dilutions of gentamicin (concentration 4 to 48 mg/L) or cefepime (concentration 40 to 5,000 mg/L) in the presence or absence of 40 mM oxaloacetate. The cultures were shaken at 37°C for 20 h and then the OD₆₀₀ was measured and pictures were taken. The MICs correspond to the lowest concentration of antibiotics that impeded growth.

Northern Blot Analysis

Total RNA of PA14, PAO1 and of PA14 and PAO1 harboring either plasmid pMMB67HE or plasmid pMMBcrcZ (grown for 20 h at 37°C in SCFM in the presence or absence of 40 mM oxaloacetate) was purified with the TRIzol reagent (Ambion) according to the manufacturer's instructions. The steady state levels of CrcZ and 5S rRNA (loading control) were determined by Northern-blotting using 4 μg of total RNA. The RNA samples were denatured for 5 min at 65°C in loading buffer containing 50% formamide, separated on a 8% 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 ³²P-end-labeled oligonucleotides [CrcZ: K3 (5′-GCT GGA GTC GTT ACG TGT TG-3′); 5S rRNA: 126 (5′-CCC CAC ACT ACC ATC GGC GAT GCG TCG-3′)]. The hybridization signals were visualized using a PhosphorImager (Molecular Dynamics). The normalization was performed with ImageQuant TL Toolbox v8.1.

RNA_{Seq}

Total RNA was prepared from two biological replicates of PA14 and PA14Δhfq after growth in SCFM to an OD₆₀₀ of 2.0. Total RNA was isolated 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. The Ribo-Zero rRNA Removal Kit (Epicenter Biotechnologies) was used to deplete rRNA from total RNA samples. Libraries were constructed using the NEBNext1Ultra™ Directional RNA Library Preparation Kit from Illumina. 100 bp single end sequence reads were

generated using the Illumina HiSeq 2000 platform at the Vienna Biocenter Campus Science Support Facility (<https://www.vbcf.ac.at/facilities/next-generation-sequencing/>). Quality control assessment, sequencing adapter removal and mapping of the samples against the PA14 reference genome (NCBI accession number NC_008463.1) were performed as described previously (Tata et al., 2016). Reads mapping to regions annotated as either rRNA or tRNA were discarded from the data. The mapped sequencing data were prepared for visualization using the ViennaNGS tool box and visualized with the UCSC Genome Browser (Wolfe et al., 2015). Reads per genes were counted using BEDTools (Quinlan and Hall, 2010) and the Refseq annotation of *P. aeruginosa* (NC_008463.1). Differential gene expression analysis was performed with DESeq (version 1) (Anders and Huber, 2010). The levels of transcripts with a fold change greater than ± 4 and a multiple testing adjusted *p*-value below 0.05 were considered to be significantly altered. The raw sequencing data were deposited in the NCBI sequence read archive (SRA) as a study under the accession number PRJNA293284.

Determination of the Proton Motive Force

PA14 and PAO1 and the corresponding *hfq* deletion strains were grown in 20 ml SCFM at 37°C to an OD₆₀₀ of 1.8–2. Then triton was added to a final concentration of 0.002% to avoid clumping. The membrane potential was measured using the dyes 3,3'-diethyloxycarbocyanine iodide [DiOC₂(3)] (BacLight™ kit; Invitrogen) and TO-PRO-3 (TO-PRO™-3 Ready Flow™ Reagent, Invitrogen) to distinguish dead from live cells during flow cytometry. The cultures were diluted 250-fold in buffer containing 100 mM Tris, 1 mM EDTA, and 80 mM NaCl (pH adjusted to 7.4 using HCl, Glasser et al., 2014). TO-PRO-3 was added according to the manufacturer's instructions and then DiOC₂(3) was added in to a final concentration of 30 μM. For depolarized controls, carbonyl cyanide 3-chlorophenylhydrazone (CCCP, dissolved in DMSO, SIGMA) was added to a final concentration of 15 μM. The samples were incubated for 15 min at room temperature and then analyzed on a BD FACS-Calibur flow cytometer. DiOC₂(3) fluorescence was measured using excitation at 488 nm and emission at 530 ± 30 nm (FL1) and 650LP nm (FL3). TO-PRO-3 fluorescence was measured using excitation at 635 nm and emission at 661 ± 13 nm (FL4). The analysis of the data was performed with FlowJo® (v10). Living cells were selected by the low fluorescence peak revealed by TO-PRO-3 staining, which were further gated on a log-scale scatter plot of side scatter (SSC) vs. forward scatter (FSC) for further analysis. Cells with a membrane potential were distinguished by an increased red/green (FL3/FL1) fluorescence ratio relative to depolarized controls. The ratiometric parameter (red-to-green ratio) was calculated according to the manufacturer's instructions [FL3–FL1 + 1.5*(#channels per decade)] and normalized to the value obtained with the wild type strain, which was set to 100%.

c-di-GMP Assay

The c-di-GMP assay was performed with strains PAO1 and PA14 and their corresponding *hfq* deletion mutants harboring plasmid pCdrA-*gfp* (ASV)^C (Rybtke et al., 2012). The strains

were grown in 25 ml SCFM supplemented with 0.01% Triton X-100 and gentamicin (30 mg/L) for plasmid maintenance in 100 ml flasks to an OD₆₀₀ of 1.8–2.0. The green fluorescence (arbitrary GFP units) as well as A₆₀₀ was measured with a plate reader (Synergy H1, BioTek). In addition, PAO1, PAO1Δ*hfq*, PA14, and PA14Δ*hfq* without plasmid were grown in the same medium to determine auto-fluorescence of the strains (blank). The corresponding auto-fluorescence was then subtracted from the green fluorescence values of each biological replicate. These values were normalized to the corresponding A₆₀₀ values.

Biofilm Assay

Static-culture biofilm assays were performed in duplicate in 5 ml round bottom polystyrene tubes (Falcon). Cells were grown in SCFM for 48 h. The biofilms were washed with water and stained with 0.1% 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 was photometrically measured at 595 nm (A₅₉₅).

RESULTS AND DISCUSSION

The Susceptibility Toward Several Classes of Antibiotics Is Increased in *P. aeruginosa* *hfq* Deletion Strains

To gain better insights into the role of Hfq in antibiotic resistance in *Pseudomonas aeruginosa*, we first determined the minimal inhibitory concentration (MIC) of seven different classes of clinically relevant antibiotics for PAO1 and PAO1Δ*hfq* (Table 1) and for the clinical strain PA14 and the isogenic *hfq* deletion mutant PA14Δ*hfq* (Table 2). The strains were grown in SCFM, which approximates to the conditions of the CF lung. When compared to the corresponding wild-type strains, the *hfq* deletion mutants showed a higher susceptibility toward all tested antibiotics regardless of whether a high inoculum (4×10^9 cells) or a low inoculum (2×10^6 cells) was seeded on the plates (Tables 1, 2). The largest differences were observed with cefepime and fosfomycin, which interfere with cell wall synthesis, and with gentamicin and tetracycline, which inhibit protein synthesis. The MICs for the same antibiotics were also tested for PAO1 and PAO1Δ*hfq* in LB-medium (Supplementary Table S1). As observed in SCFM, PAO1, and PAO1Δ*hfq* showed the same pattern of sensitivity toward the studied antibiotics. However, as these growth-dependent assays could reflect the impact of the *hfq* deletion on the ability of the respective strains to grow and divide, we also tested whether an increased susceptibility to an antibiotic is observed with PA14Δ*hfq* grown to early stationary phase. PA14 and PA14Δ*hfq* were grown in SCFM to an OD₆₀₀ of 2.0. Then, to aliquots of 180 μl culture either 20 μl of SCFM or 20 μl of SCFM containing gentamicin to a final concentration of 2 mg/L were added. For CFU determination, samples were withdrawn at an OD₆₀₀ of 2.0 (initial inoculum) and after 18 h of incubation in the presence or absence of 2 mg/L gentamicin. When compared to the wild type, the *hfq* deletion mutant showed a 10-fold reduction in the CFU (Supplementary Figure S1). Thus, the increased sensitivity of the *hfq* deletion mutants appeared to be independent of growth.

TABLE 1 | Susceptibility of PAO1 and PAO1 Δhfq toward different classes of antibiotics in SCFM medium.

Classes/Subclass	Antibiotic	Mode of action	MIC	PAO1	PAO1 Δhfq	PAO1	PAO1 Δhfq
SCFM					High inoculum ^a	Low inoculum ^b	
β -LACTAM ANTIBIOTICS							
Cephems/Cephalosporins IV	Cefepime	Cell wall	mg/L	2/3	0.75/0.75	1/1	0.5/0.5
Penems/Carbapenems	Imipenem	Cell wall	mg/L	1/1	0.5/0.5	1/1	0.5/0.5
NON- β -LACTAM ANTIBIOTICS							
Aminoglycosides	Gentamicin	Protein synthesis	mg/L	8/8	2/2	16/8	2/2
Fluoroquinolones	Ciprofloxacin	DNA metabolism	mg/L	0.25/0.25	0.12/0.12	0.12/0.12	0.06/0.12
Fosfomycins	Fosfomycin	Cell wall	mg/L	>1024/>1024	64/64	>1024/>1024	4/2
Lipopeptides/Polymixins	Colistin	Cell membrane	mg/L	3/4	2/3	6/4	3/2
Tetracyclines	Tetracycline	Protein synthesis	mg/L	128/128	32/32	16/16	8/8

/, Results of two independent experiments; >, more resistant than the highest concentration tested.

^aHigh inoculum, corresponds to 4×10^9 CFU/mL.

^bLow inoculum, corresponds to 2×10^6 CFU/mL.

TABLE 2 | Susceptibility of PA14 and PA14 Δhfq toward different classes of antibiotics in SCFM medium.

Classes/Subclass	Antibiotic	Mode of action	MIC	PA14	PA14 Δhfq	PA14	PA14 Δhfq
SCFM				High inoculum ^a		Low inoculum ^b	
β -LACTAM ANTIBIOTICS							
Cephems/Cephalosporins IV	Cefepime	Cell wall	mg/L	2/3	1.5/1.5	0.75/0.75	0.5/0.5
Penems/Carbapenems	Imipenem	Cell wall	mg/L	1/1	0.5/0.5	0.5/1	0.25/0.25
NON- β -LACTAM ANTIBIOTICS							
Aminoglycosides	Gentamicin	Protein synthesis	mg/L	8/8	2/2	4/4	2/1
Fluoroquinolones	Ciprofloxacin	DNA metabolism	mg/L	0.25/0.25	0.12/0.12	0.12/0.06	0.06/0.06
Fosfomycins	Fosfomycin	Cell wall	mg/L	> 1024/> 1024	12/6	384/384	1/2
Lipopeptides/Polymixins	Colistin	Cell membrane	mg/L	12/12	4/4	2/3	1.5/1.5
Tetracyclines	Tetracycline	Protein synthesis	mg/L	32/32	8/16	64/64	16/16

/, Results of two independent experiments; >, more resistant than the highest concentration tested.

^aHigh inoculum, corresponds to 4×10^9 CFU/mL.

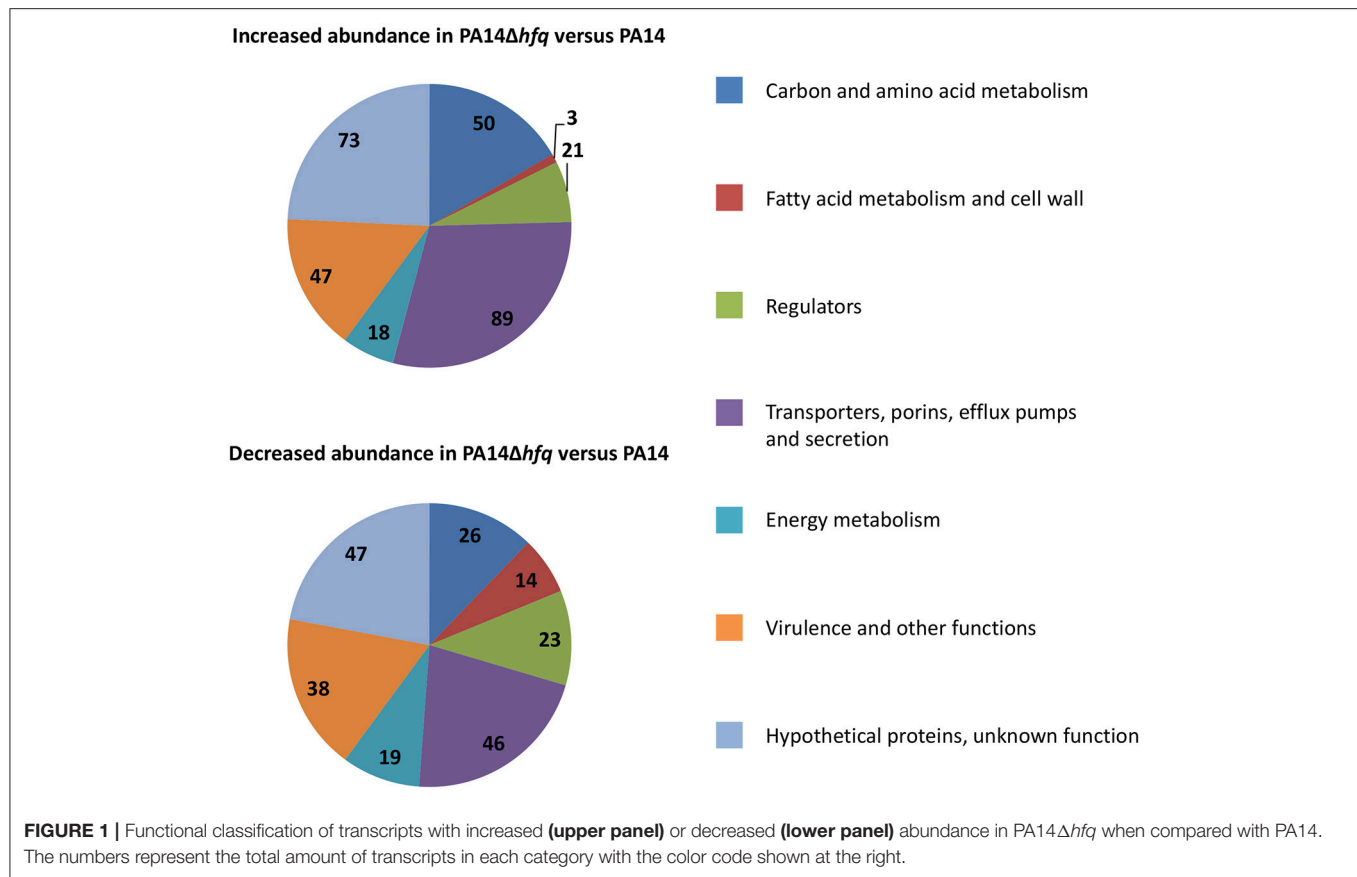
^bLow inoculum, corresponds to 2×10^6 CFU/mL.

Hfq Controls Functions Associated With Antibiotic Resistance

Next, we asked whether Hfq impacts on transcripts the regulation or mutation of which can interfere with the susceptibility to different antibiotics. As the sensitivity pattern of the wild-type and *hfq* mutant strains toward the different antibiotics was independent of the inoculum size (Tables 1, 2), a comparative RNA_{Seq} based transcriptome analysis was performed with PA14 and PA14 Δhfq grown in SCFM to an OD₆₀₀ of 1.8–2.0 (early stationary phase). A *P*-value (adjusted for multiple testing) of 0.05 was set as a threshold for significance. To select predominantly for transcripts that are regulated by Hfq, only transcripts with a change in abundance (fold-change) of ± 4 were considered. Applying these criteria, 301 transcripts were up-regulated and 213 transcripts were down-regulated in PA14 Δhfq when compared with PA14 (Supplementary Tables S2, S3). Next, the transcripts were clustered into 7 different categories (Figure 1) based on previous functional characterizations in *P. aeruginosa* or similar characterized genes in other bacteria using

the *Pseudomonas* genome database (Winsor et al., 2016). Genes encoding hypothetical proteins within operons (Wurtzel et al., 2012) with partly known functions were placed together. One of the largest group (23% in total) represented transcripts encoding “hypothetical proteins with unknown function.”

50 transcripts with increased and 28 transcripts with decreased abundance are related to “carbon and amino acid metabolism.” The high number of up-regulated genes within this category can be rationalized with Hfq being the major regulator of CCR in *P. aeruginosa* (Sonnleitner and Bläsi, 2014). It has been reported that C-sources can affect antibiotic resistance (Walker and Durham, 1975; Conrad et al., 1979; Barraud et al., 2013). However, the molecular mechanism underlying this phenomenon remained largely elusive. Meylan et al. (2017) suggested a link between central carbon metabolism and aminoglycoside sensitivity. While fumarate potentiated tobramycin susceptibility, glyoxylate promoted tolerance. This was explained by either activating (fumarate) or repressing (glyoxylate) the TCA cycle, resulting in differences in the



proton motive force (PMF), and thus differences in tobramycin uptake. Our RNA_{seq} analysis revealed elevated transcript levels of *sdhC/D*, encoding succinate dehydrogenase, *acnB*, encoding aconitate hydratase, and *acsL*, encoding acetyl-CoA synthetase, in PA14Δhfq when compared with PA14, which might suggest an up-regulation of the TCA cycle in PA14Δhfq (**Supplementary Table S2**).

Related to carbon metabolism, 3 and 14 up- and downregulated genes, respectively, clustered in the category “fatty acid metabolism and cell wall.” Among those *rfaD*, an ortholog of *Burkholderia cenocepacia* *hldD* gene, was 237.9-fold down-regulated in PA14Δhfq when compared with PA14 (**Supplementary Table S3**). The *rfaD/hldD* functions are involved in modification of heptose sugars prior to their incorporation into the LPS core (Raetz and Whitfield, 2002). It was shown for *B. cenocepacia* that deletion of *hldD* together with *hldA* resulted in short lipidA-core oligosaccharides and higher sensitivity to polymyxin B (Loutet et al., 2006). In addition, *orfM* and *orfN*, which are orthologs of PAO1 *wbpK* and *wbpL*, showed a 6.7 and 6.2-fold reduced abundance, respectively, in the absence of Hfq (**Supplementary Table S3**). They encode proteins important for the initial step of O-polysaccharide biosynthesis. In *E. coli* the lack of the O-antigen chain of LPS results in higher sensitivity toward hydrophobic antibiotics, like erythromycin and rifampicin, as well as to hydrophilic antibiotics, like vancomycin (Hirvas et al., 1997). Furthermore, a *wbpL* mutant

of *P. aeruginosa* showed increased outer membrane permeability for the antimicrobial peptide indolicidin (Gooderham et al., 2008). One positive regulator of *wbpL* transcription is PsaA (Gooderham et al., 2008). The transcript abundance of *psaA* was 4.2-fold reduced in PA14Δhfq when compared with PA14 (**Supplementary Table S3**), which is in line with the deregulation of the *wbpL* gene. A *psaA* mutant exhibit increased susceptibility to polymyxin B (Gooderham et al., 2008), which is in agreement with a higher sensitivity to colistin in PA14Δhfq when compared with PA14.

The category with the highest number of transcripts belongs to “transporters, porins, efflux pumps, and secretion” comprising 135 transcripts, 89 and 46 of which were up- and down-regulated, respectively. Intrinsic resistance to antibiotics is mainly brought about by efflux pumps (Li et al., 1994). However, the corresponding genes encoding efflux pumps such as MexAB-OprM, MexCD-OprJ, MexEF-OprN or MexXY-OprM, which are known to be involved in resistance toward several antibiotics (Morita et al., 2001; Hocquet et al., 2006; Llanes et al., 2011; Peng et al., 2017), did not show in the RNA_{seq} analysis.

The susceptibility toward imipenem increased ~8 and ~16-fold in PA14Δhfq and PAO1Δhfq when compared to the respective parental strains (**Tables 1, 2**). The transcript abundance of *oprD*, encoding a porin responsible for carbapenem uptake (Lynch et al., 1987) was 5-fold higher in PA14Δhfq than in PA14 (**Supplementary Table S2**). It has been previously reported

that Hfq negatively controls *oprD* expression indirectly on the transcriptional and directly on the post-transcriptional level via sRNAs (Ducret et al., 2016; Zhang et al., 2017), which can explain the increased susceptibility of the *hfq* deletion strains toward imipenem (Tables 1, 2, Supplementary Table S1). Moreover, the Hfq-Crc complex appears to repress translation of *oprD* mRNA directly (P. Pusic, unpublished). This seems to be also reflected in the observation that the OprD levels were elevated in a PAO1 *crc* mutant, which was paralleled by an increased susceptibility to meropenem and imipenem (Linares et al., 2010).

Another striking result was obtained with fosfomycin. PA14 was resistant to fosfomycin (Table 2), whereas the PA14Δ*hfq* strain was sensitive. The glycerol-3-phosphate permease GlpT was shown to be the only fosfomycin transporter in *P. aeruginosa* (Castañeda-García et al., 2009). The *glpT* transcript was 4.4-fold upregulated in PA14Δ*hfq* when compared with PA14 (Supplementary Table S2), suggesting an elevated uptake of fosfomycin in PA14Δ*hfq* and increased susceptibility toward fosfomycin (Table 2). The negative regulation of *glpT* by Hfq was evident from the result obtained with a transcriptional *glpT-lacZ* reporter gene. The β-galactosidase activity conferred by the transcriptional *glpT-lacZ* fusion was increased approximately 1.5-fold in PA14Δ*hfq* when compared with PA14 (Supplementary Figure S2), suggesting an indirect effect of Hfq. A PAO1*crc*- mutant showed as well an increased sensitivity to fosfomycin and increased levels of the *glpT* transcript (Linares et al., 2010), indicating that Hfq together with Crc might control a factor involved in *glpT* expression.

Several genes important for iron uptake including the transcriptional regulator gene *pvdS* are differentially abundant in PA14Δ*hfq* when compared with PA14 (Supplementary Tables S2, S3). For example *fpvA* and *fpvB*, encoding ferropyoverdine receptors, as well as the siderophore receptor PA14_45540 were upregulated in PA14Δ*hfq*, whereas pyochelin biosynthesis and uptake genes as well as *feoAB*, encoding ferrous iron transport proteins, were down-regulated when compared with PA14, suggesting aberrant iron uptake in PA14Δ*hfq*. It has been previously shown that iron depletion in *P. aeruginosa* increases the susceptibility to certain antibiotics (Oglesby-Sherrouse et al., 2014). However, it was surprising to find many iron uptake genes to be regulated by Hfq in SCFM, as the iron concentration was 100 μM. The differential abundance of functions involved in iron metabolism in PA14Δ*hfq* might be ascribed to differential c-di-GMP levels (Frangipani et al., 2014). On the other hand, there is a link between iron availability, oxidative stress and redox state of the cell (Cornelis et al., 2011), which might be also affected by Hfq, especially since transcripts like *sodB* and *katE*, encoding superoxide dismutase and catalase, were more abundant in PA14Δ*hfq* than in PA14 (Supplementary Table S2).

The category “energy metabolism” comprises 18 and 19 transcripts with increased and decreased abundance, respectively, in PA14Δ*hfq* when compared with PA14. In the absence of Hfq, the *coxBAG-coIII* operon (encoding aa₃-type cytochrome c oxidase) is up-regulated and the *cyoABCDE*

operon (encoding bo₃-type quinol oxidase) and *ccoN2O2P2* (encoding cbb₃-type cytochrome oxidase 2) are down-regulated (Supplementary Tables S2, S3). The expression of the *coxBAG-coIII* operon is low under normal growth conditions and is induced during carbon, nitrogen and iron starvation (Kawakami et al., 2010). The *cox* promoter was found to be activated by the stationary phase sigma factor RpoS and repressed by the two component system RoxS/R (Schuster et al., 2004; Kawakami et al., 2010). As the RNA_{seq} analysis revealed increased transcript levels of *rpoS* in PA14Δ*hfq* when compared with PA14 (Supplementary Table S2), it could explain the increased expression of the *cox* operon. On the other hand, Hfq might also repress translation of the *cox* operon directly. A potential Hfq binding site (–//5'–AAAAACAACGATAAG–3'//–) is present upstream of the *coxB* start codon. The aa₃-type cytochrome c oxidase has the highest proton pumping activity, and if up-regulated under nutrient rich conditions, *cox* over-expression might cause an imbalance of energy and redox homeostasis (Arai, 2011). Aminoglycoside uptake is considered to be an energy-dependent process that requires both a high membrane potential and electron flow through the respiratory chain (Taber et al., 1987). Disruption of respiration or uncoupling of the electron transport chain (ETC) protects bacteria against aminoglycoside treatment (Bryan and Kwan, 1983; Taber et al., 1987; McCollister et al., 2011). Enhanced TCA cycle activity as well as upregulation of the *cox*-operon in the PA14Δ*hfq* strain might thus suggest an increased respiration and PMF and thus elevated aminoglycoside uptake and consequently gentamicin susceptibility.

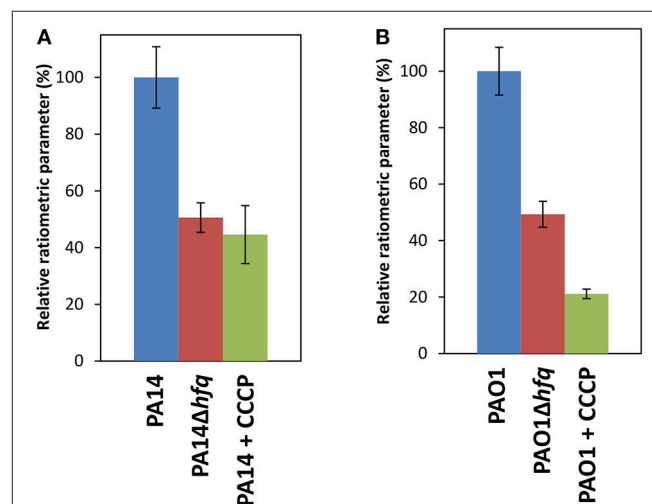


FIGURE 2 | Reduced membrane potential in the absence of Hfq. Determination of the proton motive force in PA14 (A) and in PAO1 (B) and their corresponding *hfq* deletion strains using the BacLight™ kit. The strains were grown in SCFM to an OD₆₀₀ of 1.8–2.0. The analysis was performed as described in Materials and Methods. The wild type strains and the corresponding *hfq* deletion strains are indicated by blue and red bars, respectively. Membrane depolarization in PA14 (A; green bar) and in PAO1 (B, green bar) was elicited with the addition of CCCP. The experiments were performed in duplicates. Error bars are given as standard deviation of the means over the populations of the biological replicates.

The last category of transcripts affected by Hfq includes genes related to “virulence and other functions,” some of which might be important for pathogenicity of *P. aeruginosa*, e.g., *rhlAB*, encoding rhamnosyltransferase (Abdel-Mawgoud et al., 2010) and several phenazine biosynthesis genes (Pierson and Pierson, 2010; Mavrodi et al., 2013, **Supplementary Tables S2, S3**).

Reduced Energy Metabolism in the Absence of Hfq

The RNA_{Seq} analysis suggested an increased PMF in PA14Δ*hfq* due to the up-regulation of the *cox* operon (**Supplementary Table S2**). The aa₃-type oxidase translocates the highest number of protons across the cell membrane per oxygen atom consumed (6 H⁺/O ratio) (Arai, 2011), which might allow for a partial compensation of the down-regulated operons encoding bo₃ and ccb₃₂-type oxidases (4 H⁺/O ratio) (**Supplementary Table S3**). To test whether Hfq affects the PMF and therefore the membrane potential, we used a flow cytometry based method for membrane potential measurements. This technique is based on the carbocyanine dye DiOC₂(3) (3,3'-diethyloxycarbocyanine iodide). DiOC₂(3) exhibits green fluorescence in all bacterial cells, but the fluorescence shifts toward red emission as soon as the dye molecules self-associate due to higher cytosolic concentrations that is caused by an increased membrane potential. For a better correlation between dye uptake and membrane potential, ratiometric values (the determined values of the fluorescence were divided by a parameter value representative for cell sizes) were established (Novo et al., 1999).

Unexpectedly, PA14Δ*hfq* revealed a 50% reduced ratiometric value compared to PA14, which was similar to that when the membrane potential was disrupted by carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (**Figure 2A**). Comparable results were obtained with the corresponding PAO1 strains (**Figure 2B**), except that the impact of CCCP was more pronounced (80% reduction). These results indicated that deletion of *hfq* results in a decrease of the energy metabolism, which however cannot be straightforwardly reconciled with the RNA_{Seq} analysis. However, RNA_{Seq} based transcriptome studies only reveal either direct or indirect transcriptional regulation by Hfq or reflect post-transcriptional regulation if the regulatory event impacts on transcript abundance. Thus, as shown by Grenga et al. (2017), a considerable proportion of the Hfq regulon that is controlled at the translational and post-translational level will not be reflected in the transcriptome analysis.

It has been shown that disruption of the membrane potential by CCCP resulted in increased susceptibility to colistin and ciprofloxacin in some clinical strains of *P. aeruginosa* (Adabi et al., 2015; Ni et al., 2016) and that the PMF is important for the function of MFS (major facilitator superfamily) and RND (resistance-nodulation-division) efflux pumps (Paulsen et al., 1996). Thus, the reduced PMF in the *hfq* deletion strains might interfere with antibiotic efflux, resulting in increased susceptibility to certain antibiotics, like β-lactams, fluoroquinolones, tetracycline and aminoglycosides that are exported via efflux pumps (Schweizer, 2003). The apparent reduction in energy metabolism of the *hfq* deletion strains (**Figure 2**) cannot be easily reconciled with the increased

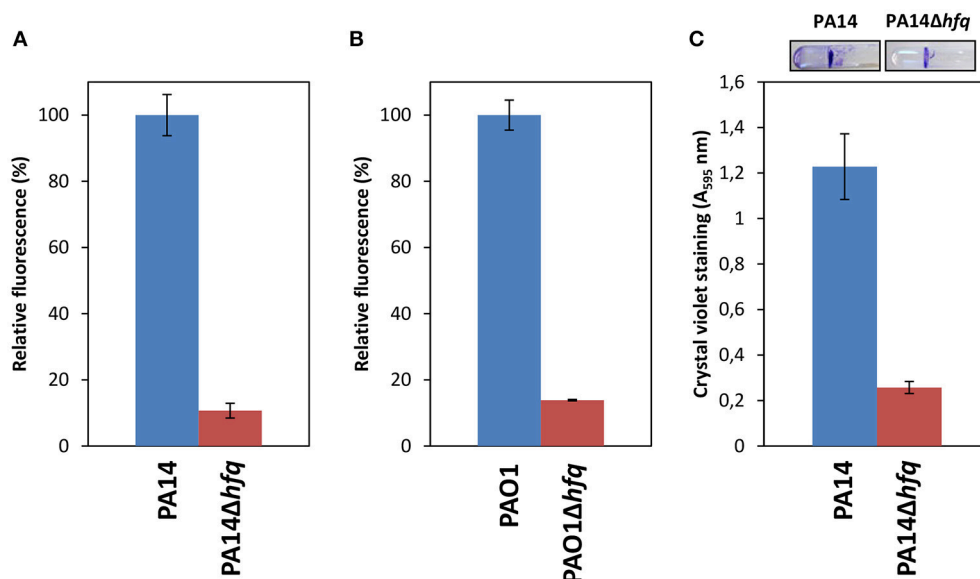


FIGURE 3 | Deletion of *hfq* results in reduced c-di-GMP levels and biofilm formation. PA14 (**A**) and PAO1 (**B**) (blue bars) and the corresponding *hfq* deletion strains (red bars) harboring plasmid pCdrA::gfp(ASV)^C, carrying a c-di-GMP sensitive transcriptional *cdrA-gfp* fusion gene, were grown in SCFM to an OD₆₀₀ of 1.8–2.0. The analysis was performed as described in Materials and Methods. (**C**) Static biofilms of PA14 and PA14Δ*hfq* were stained with crystal violet after growth in SCFM for 48 h. The stain was then eluted by ethanol and the absorption at 595 nm was measured. Representative pictures of the PA14 and PA14Δ*hfq* biofilms are shown on top. The experiments were performed in duplicate. Error bars are given as standard deviation.

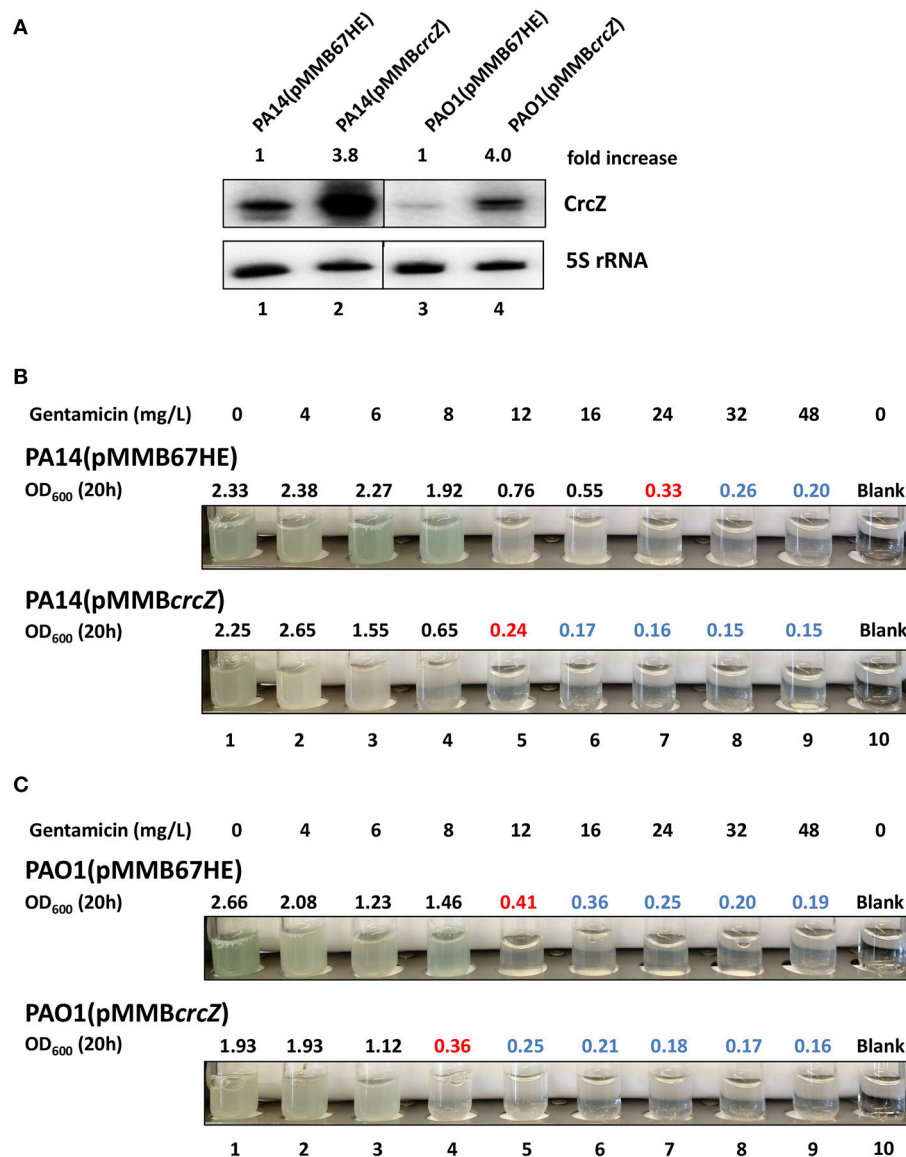


FIGURE 4 | Over-production of CrcZ results in increased susceptibility to gentamicin. **(A)** PA14 and PAO1 harboring plasmid pMMB67HE and plasmid pMMBcrrZ, respectively, were grown in SCFM for 20 h. The cells were harvested and the CrcZ levels were determined by Northern-blot analysis. 5S rRNA served as a loading control. The fold increase was calculated by dividing the signal intensity of CrcZ by the corresponding signal of 5S rRNA and normalized to the signal obtained with the respective wild type strain harboring plasmid pMMB67HE that was set to 100%. Lanes 1 and 2 contain RNA extracted from strains PA14 (pMMB67HE) and PA14 (pMMBcrrZ), respectively. Lanes 3 and 4 contain RNA extracted from strains PAO1(pMMB67HE) and PAO1(pMMBcrrZ), respectively. **(B,C)** MIC determination for gentamicin of PA14 **(B)** and PAO1 strains **(C)**. The cells harboring plasmid pMMB67HE (upper panels) and plasmid pMMBcrrZ (lower panels), respectively, were grown as described above. The different concentrations of gentamicin added are indicated on top. Pictures were taken and the OD₆₀₀ was measured 20 h after inoculation. The antibiotic concentrations in the presence of which the cells did not grow above OD₆₀₀ of 0.5 (marked in red) were considered as MIC. All OD₆₀₀ values above this gentamicin concentration are depicted in blue indicating toxicity. The experiments were performed in duplicate, revealing the same MICs. Only one representative experiment is shown.

susceptibility to gentamicin in terms of drug uptake. However, in addition to a possible impairment of the efflux pumps, the down-regulation of some LPS biosynthetic genes in PA14Δ*hfq* (Supplementary Table S3) could as well contribute to the increased susceptibility toward aminoglycosides (Hirvas et al., 1997).

Reduced c-di-GMP Levels in the Absence of Hfq

Bacterial biofilms are more resistant to antibiotics than planktonically growing cells (Ceri et al., 1999; Olsen, 2015). Most likely, there is a multitude of factors that promote biofilm-specific antibiotic tolerance/resistance (Olsen, 2015).

The secondary messenger c-di-GMP is a key signal in post-transcriptional regulation of biofilm formation and coordinates the transition from a motile to a sessile lifestyle and *vice versa* (i.e., dispersion, Valentini and Filloux, 2016; Moradali et al., 2017). A comparison of the RNA_{Seq} analysis performed in this study with the transcriptome profiles of *P. aeruginosa* subjected to high or low c-di-GMP levels (Lin Chua et al., 2017) revealed that 43% of all Hfq regulated transcripts are also c-di-GMP dependent. To determine whether Hfq impacts on the c-di-GMP levels, a transcriptional reporter gene was employed that is comprised of the c-di-GMP responsive *cdrA* promoter fused to the *gfp* (ASV)^C gene, which encodes an unstable GFP variant (Rybtke et al., 2012). As shown in **Figure 3A**, under these conditions, the reporter gene conferred ~ 60% less GFP activity in PA14Δ*hfq*(pCdrA-*gfp*(ASV)^C) when compared with PA14 (pCdrA-*gfp*(ASV)^C). A similar reduction in GFP activity of 70% was obtained with PAO1Δ*hfq* (pCdrA-*gfp*(ASV)^C) when compared with PAO1(pCdrA-*gfp*(ASV)^C) (**Figure 3B**). These results showed that the c-di-GMP levels were reduced in the *hfq* deletion strains, which was also reflected by the diminished ability of PA14Δ*hfq* to form biofilms in SCFM when compared to PA14 (**Figure 3C**).

Reduced c-di-GMP levels resulted in a mild increase of the OprD protein levels (Nicastro et al., 2014). Thus, in addition to the Hfq-mediated negative riboregulation of *oprD* (Zhang et al., 2017), the decreased c-di-GMP levels in the *hfq* deletion strains could as well contribute to the increased imipenem susceptibility. The transcriptome analysis did not identify a differential abundance in the absence of Hfq of transcripts encoding a c-di-GMP phosphodiesterase or a diguanylate cyclase. However, as discussed above, the RNA_{Seq} analysis has limitations (Grenga et al., 2017) in that translational and/or post-translational regulatory events might not be mirrored.

Harnessing Metabolic Control for Sensitization Toward Antibiotics

As the deletion of *hfq* resulted in increased susceptibility of *P. aeruginosa* to different antibiotics, we next asked whether we could harness the observed interference by CrcZ RNA of Hfq-mediated regulatory events (Pusic et al., 2016; Sonleitner et al., 2017), i.e., whether an up-regulation of CrcZ RNA results in increased antibiotic susceptibility through titration of Hfq.

To address this, PA14 and PAO1 harboring plasmid pMMB67HE (empty plasmid) and plasmid pMMB*crcZ* (*P_{tac}* driven constitutive expression of *crcZ*), respectively, were grown in SCFM medium to an OD₆₀₀ of 1.0. Then, one ml of culture was mixed with one ml of fresh SCFM containing serial dilutions of gentamicin and incubated for 20 h at 37°C. As shown in **Figure 4**, over-production of CrcZ (**Figure 4A**) resulted in an increased sensitivity of PA14 (pMMB*crcZ*) (**Figure 4B**) and PAO1(pMMB*crcZ*) (**Figure 4C**) toward the antibiotic in a manner comparable with the patterns observed for the *hfq* deletion strains (**Tables 1, 2**).

The *crcZ* promoter activity, and thus the levels of CrcZ are increased when the cells grow on non-preferred carbon sources such as oxaloacetate (OAA) or histidine (Valentini

et al., 2014). To further test whether the CrcZ levels can be correlated with antibiotic susceptibility, the growth inhibition zones obtained with PA14 and PAO1 in the presence of cefepime, colistin, ciprofloxacin and gentamicin were determined on BSM agar plates supplemented with either succinate or OAA. When compared to cells growing on succinate, PAO1 and PA14 growing on OAA were more susceptible to all tested antibiotics (**Table 3**), supporting the hypothesis that C-source dependent antibiotic susceptibility can be linked to increased CrcZ levels and thus enhanced sequestration of Hfq.

Next, we asked whether the CrcZ levels and thus antibiotic susceptibility can be increased in complex medium by adding OAA. PA14 and PAO1 were grown in SCFM medium to an OD₆₀₀ of 1.0. Then, one ml of culture was mixed with one ml of fresh SCFM with or without 40 mM OAA and incubated for 20 h at 37°C. As shown in **Figure 5A**, the levels of CrcZ increased upon addition of OAA to SCFM in PA14 and PAO1. Concurrently, the MIC for gentamicin changed from 16 mg/L (without OAA) to 6 mg/L (with OAA) in PA14 (**Figure 5B**) and from 24 mg/L (without OAA) to 12 mg/L (with OAA) in PAO1 (**Figure 5C**), indicating a significant increase in susceptibility for gentamicin upon addition of OAA. A similar increase in the susceptibility toward cefepime was also observed for PAO1 when OAA was added to the growing culture (change in the MIC from 2,500 to 310 mg/L in the presence of OAA, **Supplementary Figure S3**). Taken together, these data hold promise that induced synthesis of CrcZ can be harnessed to increase the susceptibility of or re-sensitize *P. aeruginosa* toward different antibiotics by means of sequestration of Hfq.

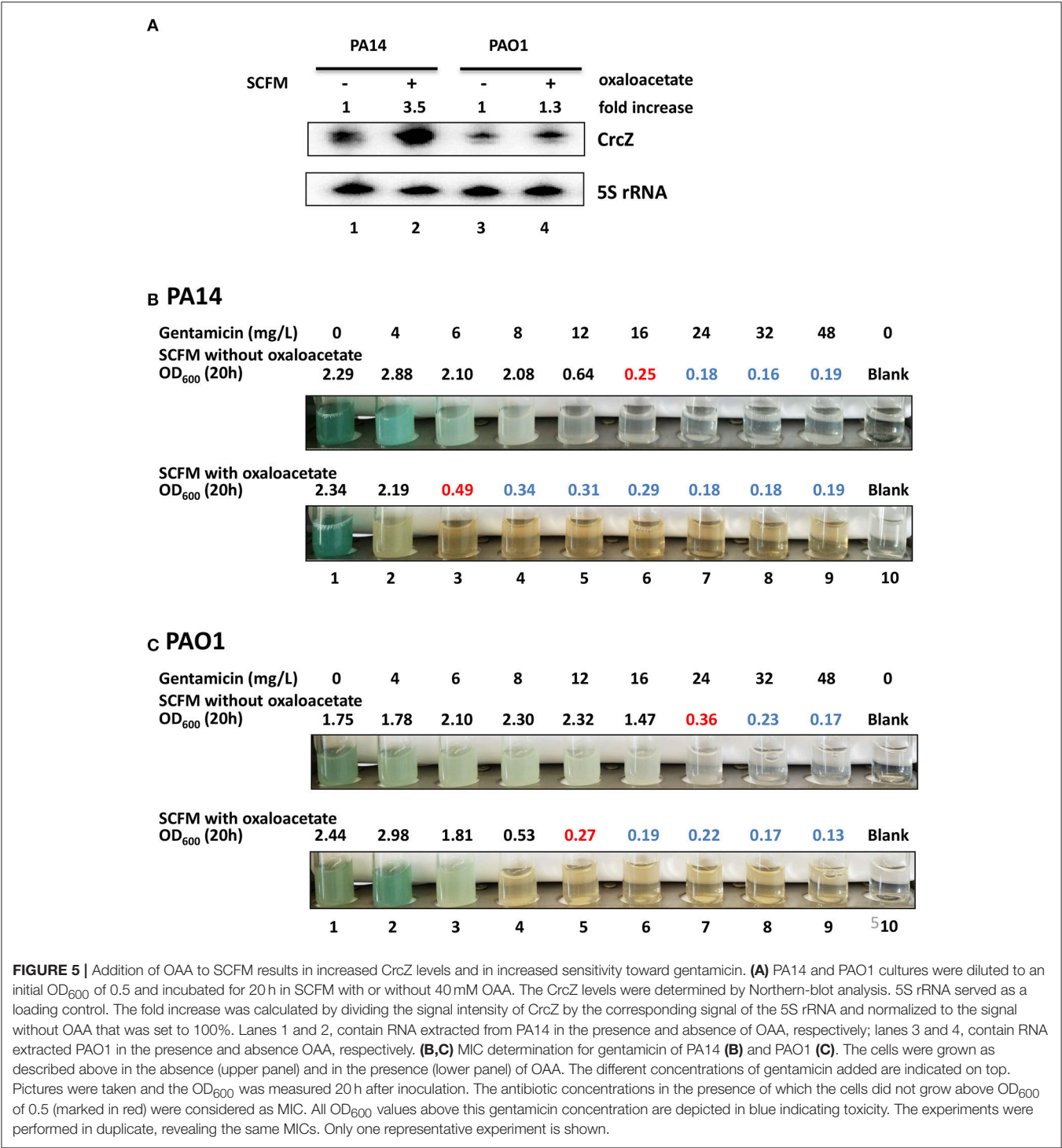
CONCLUSIONS AND PERSPECTIVES

In this study we have shown that Hfq impacts on energy metabolism and c-di-GMP levels. Thus, the absence of Hfq seems to impose a general fitness burden which is also apparent from the somewhat impaired growth of the *hfq* deletion strains (not shown). It was shown that dormant *P. aeruginosa* exhibit an impaired aminoglycoside uptake. This phenomenon called phenotypic tolerance is related to persistence of bacteria after antibiotic treatment (Martínez and Rojo, 2011). Interestingly, *P. aeruginosa* can be re-sensitized toward aminoglycosides

TABLE 3 | The sensitivity toward several antibiotics is increased during growth on oxaloacetate.

Antibiotic	Conc.(μg)	PA14 (%)		PAO1 (%)	
BSM		Succinate	Oxaloacetate	Succinate	Oxaloacetate
Cefepime	60	100 ± 6	107 ± 6	100 ± 3	137 ± 3
Colistin	25	100 ± 5	130 ± 6	100 ± 5	126 ± 6
Ciprofloxacin	5	100 ± 5	112 ± 4	100 ± 3	125 ± 2
Gentamicin	120	100 ± 0	119 ± 0	100 ± 3	131 ± 4

The diameter of the growth inhibition zone for each antibiotic in BSM-succinate was set to 100 %. The inhibition zones obtained during growth on oxaloacetate were normalized to the diameter revealed in BSM-succinate. The data are derived from two independent experiments as mean ± standard deviation.



by metabolic stimulation (Meylan et al., 2017). However, in contrast to persister cells, the slower growing *hfq* deletion strains were more susceptible to all tested antibiotics including aminoglycosides.

The transcriptome analysis revealed that Hfq governs the expression of many functions, which affect antibiotic susceptibility. Thus, physiological changes on the one hand and the regulation of specific antibiotic resistance determinants on the other hand seem to collectively contribute to the observed increased susceptibility to antibiotics. The specific regulatory mechanisms exerted by Hfq on resistance genes/determinants being it via riboregulation or direct translational repression remain to be elucidated for the individual functions in detail. However, it is striking that a PAO1 *crc*-deficient mutant mirrored

the increased susceptibility phenotypes of the PAO1 Δ hfq strain in terms of increased sensitivity toward aminoglycosides, beta-lactams, fosfomycin and polymyxin B (Linares et al., 2010). Hence, it is conceivable that Hfq in conjunction with Crc directly regulates several functions conferring susceptibility at the post-transcriptional level. Thus, the Hfq-Crc complex appears to be a promising drug target to achieve a higher efficacy of treatments with a broad spectrum of different antibiotics.

Hfq is titrated by the regulatory RNA CrcZ, which abrogates its function in Hfq-Crc-mediated translational repression during CCR as well as in riboregulation (Sonnleitner and Bläsi, 2014; Sonnleitner et al., 2017). Here, we have provided a proof of principle that metabolic rewiring by induced synthesis of CrcZ RNA during growth in complex medium can increase the efficacy of gentamicin and cefepime. It is currently unknown, which signal is sensed by the CbrA/B two component system that controls CrcZ synthesis. The elucidation of the signal might thus permit induced synthesis of CrcZ in the presence of a given antibiotic. This might not only allow to lower the effective dose of antibiotics but also to re-sensitize *P. aeruginosa* strains toward certain antibiotics, e.g., for fosfomycin. In this context it is worth noting that PAO1 was recently shown to be more susceptible to fosfomycin under hypoxic conditions due to overexpression of *glpT* in an ANR dependent manner (Hirakawa et al., 2018). As CrcZ was the predominant RNA bound to Hfq in anoxic biofilms (Pusic et al., 2016) and Hfq impacts on *glpT* expression (Supplementary Figure S2) both might as well contribute to anaerobic regulation of *glpT*. Moreover, we have shown that the absence of Hfq results in less efficient aerobic (this study) and anoxic biofilm formation (Pusic et al., 2016).

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Thus, it appears worthwhile testing whether inactivation of Hfq might additionally increase the susceptibility of biofilms toward antibiotics.

AUTHOR CONTRIBUTIONS

UB, ES: Conceived and designed the experiments; ES, PP, BK, DH, and AR: Performed the experiments; ES, PP, MW, and UB: Analyzed the data; ES, PP, and UB: Wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.02709/full#supplementary-material>

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5.3.1. Supplementary Figures and Tables

Supplementary Material

Harnessing Metabolic Regulation to increase Hfq-dependent Antibiotic Susceptibility in *Pseudomonas aeruginosa*

Petra Pusic⁺, Elisabeth Sonnleitner*⁺, Beatrice Krennmayr, Dorothea A. Heitzinger, Michael T. Wolfinger, Armin Resch and Udo Bläsi*

⁺ These authors contributed equally to this work

*** Correspondence:**

Elisabeth Sonnleitner: elisabeth.sonnleitner@univie.ac.at

Udo Bläsi: udo.blaesi@univie.ac

1 Supplementary Figures and Tables

1.1 Supplementary Tables

Supplementary Table S1. Susceptibility of PAO1 and PAO1 Δhfq towards different classes of antibiotics in LB medium.

Classes/Subclass	Antibiotic	Mode of action	MIC	PAO1	PAO1Δhfq	PAO1	PAO1Δhfq
SCFM					High inoculum ^a	Low inoculum ^b	
β-Lactam antibiotics							
Cephems/Cephalosporins IV	Cefepime	cell wall	mg/L	1.5/1.5	1.5/1.5	0.5/0.38	0.5/0.38
Penems/Carbapenems	Imipenem	cell wall	mg/L	2/2	1/1	1/1	0.5/0.25
Non-β-Lactam antibiotics							
Aminoglycosides	Gentamicin	protein synthesis	mg/L	4/4	0.25/0.5	2/2	0.5/0.25
Fluoroquinolones	Ciprofloxacin	DNA metabolism	mg/L	0.12/0.12	0.03/0.06	0.06/0.06	0.008/0.008
Fosfomycins	Fosfomycin	cell wall	mg/L	>1024/>1024	24/16	46/46	32/32
Lipopeptides/Polymixins	Colistin	cell membrane	mg/L	1/1.5	0.38/0.5	0.75/0.75	0.094/0.064
Tetracyclines	Tetracycline	protein synthesis	mg/L	16/16	8/8	8/16	4/4

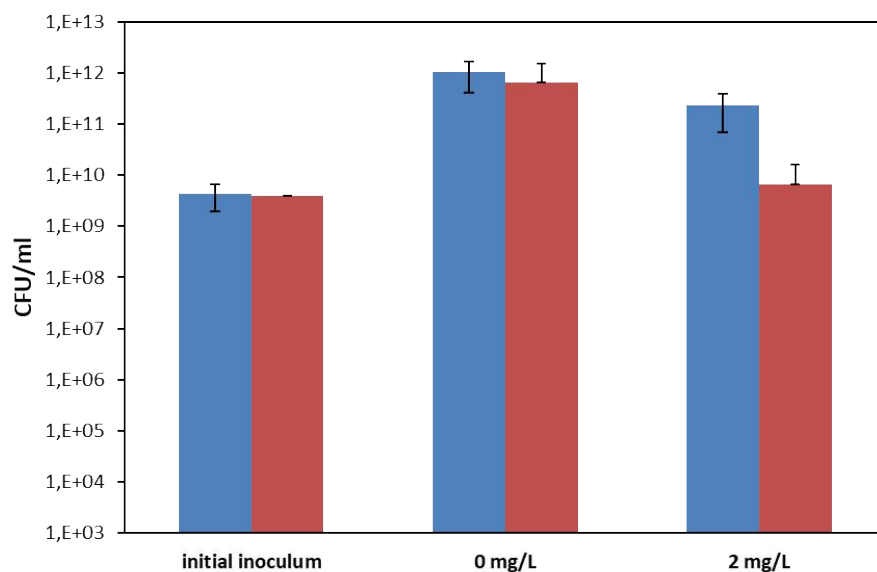
/, results of two independent experiments; >, more resistant than the highest concentration tested.

^{a, b} See Table 1.

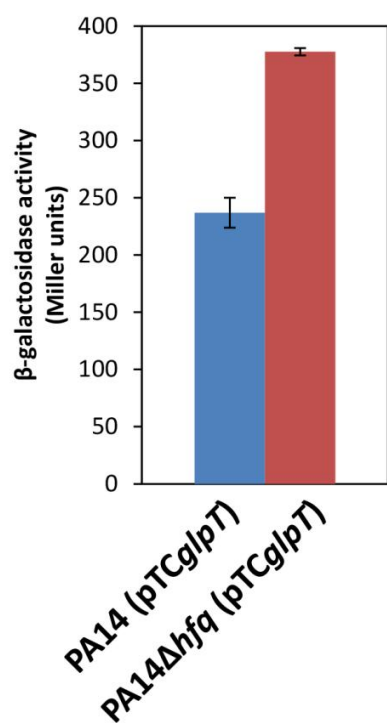
Supplementary Table S2 Transcripts with increased abundance in PA14 Δhfq versus PA14.

Supplementary Table S3 Transcripts with decreased abundance in PA14 Δhfq versus PA14.

1.2 Supplementary Figures

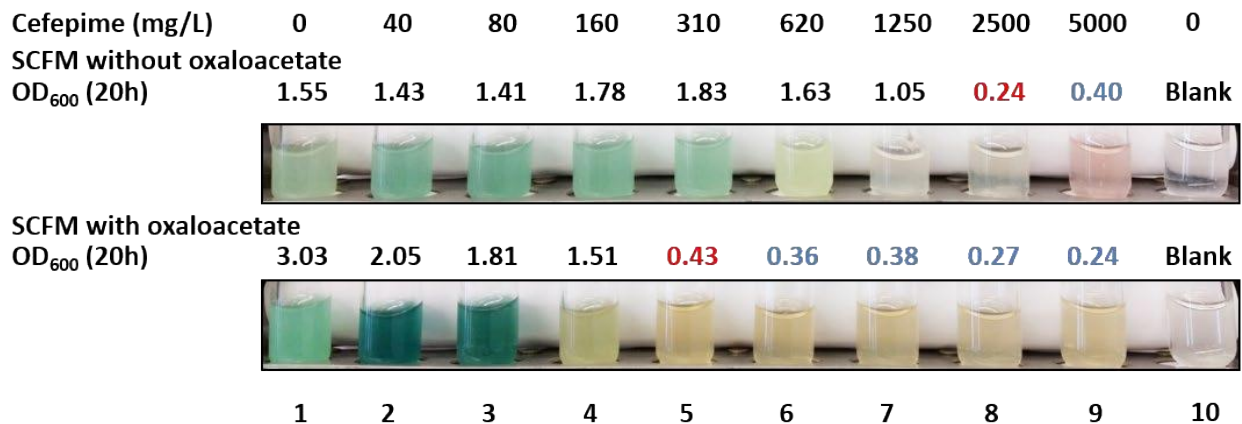


Supplementary Figure S1 Viability of early stationary phase cells of PA14 and PA14 Δhfq after treatment with gentamicin. Determination of the CFUs of PA14 (blue bars) and PA14 Δhfq (red bars) at an OD₆₀₀ of 2.0 (initial inoculum) and in the absence (0 mg/L) or presence (2 mg/L) of gentamicin after 18 h of static growth in SCFM.



Supplementary Figure S2 The transcription of *glpT* is increased in the absence of Hfq. The strains PA14 (pTC*glpT*) (blue bar) and PA14Δ*hfq* (pTC*glpT*) (red bar) were grown in SCFM. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β-galactosidase values conferred by the plasmid pTC*glpT* encoded transcriptional *glpR-lacZ* fusion gene in the presence or absence of *hfq*. The error bars represent standard deviations from three independent experiments.

PAO1



Supplementary Figure S3 Addition of OAA to SCFM results in increased sensitivity of PAO1 towards cefepime. The PAO1 culture was diluted to an initial OD₆₀₀ of 0.5 and incubated for 20 h in SCFM with or without 40 mM OAA in the presence of different cefepime concentrations as indicated on top. Pictures were taken and the OD₆₀₀ was measured 20 h after inoculation. The antibiotic concentrations in the presence of which the cells did not grow above OD₆₀₀ of 0.5 (marked in red) were considered as MIC. All OD₆₀₀ values obtained above this cefepime concentration are depicted in blue indicating toxicity. The experiments were performed in duplicate, revealing the same MICs. Only one representative experiment is shown.

Supplementary Table S2. Transcripts with increased abundance in PA14Δ*hfg* versus PA14

PA14_id ^{a,b}	Gene	Description ^a	foldChange	padj	Gene homologs in PAO1 ^b	Gene PAO1	c-di-GMP dep. ^c
Carbon and amino acid metabolism							
PA14_01910	<i>pcaG</i>	protocatechuate 3,4-dioxygenase subunit alpha	6,1	3,6E-13	PA0154	<i>pcaG</i>	
PA14_01810		oxidoreductase	4,2	2,3E-17	PA0147		
PA14_02760		CoA transferase, subunit A	15,7	2,1E-44	PA0226		X
PA14_02770	<i>pcaJ</i>	CoA transferase subunit B	5,6	1,5E-17	PA0227		X
PA14_06190	<i>psfA</i>	glutathione S-transferase	4,2	1,1E-21	PA0473		
PA14_10230	<i>adh</i>	2,3-butanediol dehydrogenase	40,4	1,2E-121	PA4153		X
PA14_10240	<i>acoC</i>	branched-chain alpha-keto acid dehydrogenase subunit E2	43,3	1,8E-123	PA4152		X
PA14_10250	<i>acoB</i>	acetoin catabolism protein AcoB	96,3	1,9E-161	PA4151	<i>acoB</i>	X
PA14_10260	<i>acoA</i>	dehydrogenase E1 component	100,6	9,0E-165	PA4150		X
PA14_10270	<i>acoX</i>	hypothetical protein	214,4	3,0E-157	PA4149		X
PA14_10280		short-chain dehydrogenase	316,2	4,1E-164	PA4148		X
PA14_10630	<i>hpcC</i>	5-carboxy-2-hydroxymuconate semialdehyde dehydrogenase	4,4	3,2E-20	PA4123	<i>hpcC</i>	
PA14_10640		hypothetical protein	14,3	4,9E-39	PA4122		
PA14_10990	<i>hpaC</i>	4-hydroxyphenylacetate 3-monooxygenase small subunit	5,3	3,5E-07	PA4092	<i>hpaC</i>	
PA14_14730	<i>iscS</i>	cysteine desulfurase	6,0	7,0E-38	PA3814	<i>iscS</i>	X
PA14_17960	<i>glpK</i>	glycerol kinase	6,0	1,7E-36	PA3582	<i>glpK</i>	X
PA14_18120	<i>mmsA</i>	methylmalonate-semialdehyde dehydrogenase	37,0	8,5E-124	PA3570	<i>mmsA</i>	X
PA14_18140	<i>mmsB</i>	3-hydroxyisobutyrate dehydrogenase	29,4	6,7E-109	PA3569	<i>mmsB</i>	X
PA14_18150	<i>acsL</i>	acetyl-CoA synthetase	114,8	1,9E-186	PA3568		X
PA14_18830		adenosuccinate lyase	5,9	5,2E-18	PA3517		
PA14_18850		adenosuccinate lyase	5,8	3,1E-22	PA3516		
PA14_20560	<i>amiE</i>	acylamide amidohydrolase	27,5	1,1E-100	PA3366	<i>amiE</i>	
PA14_20570		chaperone	5,7	7,9E-25	PA3365	<i>amiB</i>	
PA14_23750	<i>leuC</i>	isopropylmalate isomerase large subunit	8,1	2,0E-47	PA3121	<i>leuC</i>	
PA14_28180		hypothetical protein	5,6	4,5E-30	PA2776	<i>pauB3</i>	
PA14_31820	<i>hisC</i>	aminotransferase	4,5	1,4E-16	PA2531		X
PA14_32985	<i>gcvH2</i>	glycine cleavage system protein H	4,7	7,0E-27	PA2446	<i>gcvH2</i>	X
PA14_33000	<i>gcvP2</i>	glycine dehydrogenase	6,0	1,4E-37	PA2445	<i>gcvP2</i>	
PA14_35520	<i>bkdA2</i>	2-oxoisovalerate dehydrogenase subunit beta	4,8	1,6E-29	PA2248	<i>bkdA2</i>	X
PA14_35530	<i>bkdA1</i>	2-oxoisovalerate dehydrogenase subunit alpha	12,3	1,6E-29	PA2247	<i>bkdA1</i>	X
PA14_36370		carboxylate-amine ligase	4,6	2,1E-18	PA2181		
PA14_36570	<i>glgA</i>	glycogen synthase	4,7	5,8E-26	PA2165		X
PA14_36580		glycosyl hydrolase	4,3	5,9E-23	PA2164		X
PA14_39590	<i>metE</i>	5-methyltetrahydropteroyltrimethylglutamate/homocysteine S-methyltra	5,3	1,7E-27	PA1927	<i>metE</i>	
PA14_41470	<i>acnB</i>	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	4,3	8,8E-25	PA1787	<i>acnB</i>	X
PA14_43780		oxidoreductase	4,1	3,0E-11	PA1602		
PA14_43790		aldehyde dehydrogenase	4,9	8,1E-20	PA1601		X
PA14_44050	<i>sdhD</i>	succinate dehydrogenase (D subunit)	4,4	1,4E-17	PA1582	<i>sdhD</i>	
PA14_53940	<i>prpB</i>	2-methylisocitrate lyase	4,6	5,6E-27	PA0796	<i>prpB</i>	X
PA14_64520		putative bacterioferritin	16,0	9,0E-82	PA4880		X
PA14_64850		ornithine cyclodeaminase	4,5	3,0E-24	PA4908		X
PA14_67320	<i>hutH</i>	histidine ammonia-lyase	4,3	1,2E-15	PA5098	<i>hutH</i>	X
PA14_44060	<i>sdhC</i>	succinate dehydrogenase, cytochrome b556 subunit	4,4	4,3E-21	PA1581	<i>sdhC</i>	
PA14_69990	<i>dadX</i>	alanine racemase	9,4	7,3E-52	PA5302	<i>dadX</i>	
PA14_70640	<i>rubA1</i>	rubredoxin 1	10,8	2,9E-46	PA5351	<i>rubA1</i>	
PA14_70670	<i>glcF</i>	glycolate oxidase iron-sulfur subunit	5,3	1,2E-15	PA5353	<i>glcF</i>	X
PA14_70680	<i>glcE</i>	glycolate oxidase FAD binding subunit	6,0	2,0E-14	PA5354	<i>glcE</i>	X
PA14_70690	<i>glcD</i>	glycolate oxidase subunit GlcD	5,8	2,6E-21	PA5355	<i>glcD</i>	X
PA14_71460	<i>glyA1</i>	serine hydroxymethyltransferase	5,1	3,1E-11	PA5415	<i>glyA1</i>	
PA14_71470	<i>soxB</i>	sarcosine oxidase beta subunit	5,3	3,4E-12	PA5416	<i>soxB</i>	
Fatty acid metabolism and cell wall							
PA14_25670	<i>acpP</i>	acyl carrier protein	4,3	8,9E-15	PA2966	<i>acpP</i>	
PA14_28310		enoyl-CoA hydratase	4,8	1,1E-14	PA2767		X
PA14_64990		putative cholinesterase	4,3	2,7E-16	PA4921	<i>choE</i>	
Regulators							
PA14_01840		RNA polymerase ECF-subfamily sigma-70 factor	4,4	9,1E-19	PA0149		X
PA14_01930	<i>pcaR</i>	transcriptional regulator PcaR	5,4	2,5E-16	PA0155	<i>pcaR</i>	
PA14_07970		putative suppressor of type III secretion system	8,9	2,9E-28	PA0612	<i>ptrB</i>	
PA14_07980		hypothetical protein	5,2	2,4E-26	PA0613		
PA14_10290	<i>acoR</i>	transcriptional regulator AcoR	13,2	6,1E-71	PA4147	<i>acoR</i>	X
PA14_10940		AraC family transcriptional regulator	4,5	5,4E-24	PA4094		
PA14_14710		Rrt2 family protein	5,3	3,5E-33	PA3815	<i>iscR</i>	X
PA14_17480	<i>rpoS</i>	RNA polymerase sigma factor RpoS	6,5	6,7E-41	PA3622	<i>rpoS</i>	
PA14_18200		LysR family transcriptional regulator	4,3	3,6E-19	PA3565		
PA14_27230		MarR family transcriptional regulator	31,0	1,3E-107	PA2849	<i>ohrR</i>	
PA14_32060	<i>xylS</i>	transcriptional regulator XylS	14,8	2,6E-44	PA2519	<i>xylS</i>	
PA14_33260	<i>pvdS</i>	extracytoplasmic-function sigma-70 factor	8,2	2,5E-47	PA2426	<i>pvdS</i>	
PA14_36880		ompetence-damaged protein	4,4	4,3E-13	PA2141		X
PA14_38250		putative transcriptional regulator	6,4	9,4E-30	PA2032		
PA14_38930	<i>glpR</i>	transcriptional regulator	6,7	3,6E-17	PA1978	<i>erbR</i>	
PA14_39800		ECF subfamily RNA polymerase sigma-70 factor	5,2	2,8E-29	PA1912	<i>femI</i>	X
PA14_39810		transmembrane sensor	4,0	1,7E-11	PA1911	<i>femR</i>	X
PA14_39980	<i>qscR</i>	transcriptional regulator	48,7	2,2E-73	PA1898	<i>qscR</i>	
PA14_53920		putative transcriptional regulator	5,5	4,0E-28	PA0797		X
PA14_55730		AraC family transcriptional regulator	4,5	2,9E-20	PA4288		
PA14_71070		AraC family transcriptional regulator	16,3	3,4E-78	PA5380	<i>gbdR</i>	
Transporters, porins, efflux pumps and secretion							
PA14_09970	<i>fpvB</i>	type I ferritinoverdine receptor, FpvB	10,0	3,9E-55	PA4168	<i>fpvB</i>	
PA14_00560	<i>exoT</i>	exoenzyme T	15,4	5,7E-78	PA0044	<i>exoT</i>	X
PA14_01020		putative type VI secretion system protein	5,3	1,5E-31	PA0084	<i>tssC1</i>	X
PA14_01060		putative type VI secretion system protein	6,7	1,2E-20	PA0087	<i>tssE1</i>	
PA14_01070		putative type VI secretion system protein	5,6	7,1E-24	PA0088	<i>tssF1</i>	X
PA14_05790		putative transporter	886,3	1,6E-274	PA0443		
PA14_10110		putative transporter	119,6	1,4E-179	NA		
PA14_10300		putative efflux protein	12,1	3,7E-39	NA		
PA14_10440		probable porin	10,8	2,9E-36	PA4137		
PA14_13990		probable amino acid ABC transporter	4,2	2,1E-23	PA3865		
PA14_14330		putative Pseudomonas chaperone for ExoS	38,6	1,1E-80	PA3842	<i>spcS</i>	X
PA14_15200		putative transporter	5,8	1,2E-12	PA3779		

PA14_16410		probable major facilitator superfamily (MFS) transporter	81,1	1,1E-157	PA3709		
PA14_22340		hypothetical protein	17,8	2,6E-69	PA3235		X
PA14_22350	<i>actP</i>	acetate permease	38,2	1,7E-125	PA3234		X
PA14_24790	<i>opdQ</i>	outer membrane porin	66,0	7,8E-153	PA3038	<i>opdQ</i>	
PA14_26050		putative transporter	16,7	5,8E-74	PA2938		X
PA14_28400	<i>oprQ</i>	outer membrane OprD family porin	17,2	1,0E-83	PA2760	<i>oprQ</i>	
PA14_29200		putative type VI secretion system protein	5,8	1,2E-20	PA2702	<i>tse2</i>	
PA14_29390		putative type VI secretion system protein	4,1	3,6E-22	PA2685	<i>vgrG4</i>	
PA14_33680	<i>fpvA</i>	ferrityoverdine receptor	9,7	2,6E-57	PA2398	<i>fpvA</i>	
PA14_34050		putative type VI secretion system protein	8,8	2,5E-50	PA2366	<i>hsiC3</i>	X
PA14_34070		putative type VI secretion system protein	11,7	5,9E-60	PA2365	<i>hsiB3</i>	X
PA14_34800		putative amino acid transporter	9,7	1,9E-44	PA2306	<i>ambA</i>	X
PA14_36120		putative MFS transporter	5,5	8,8E-17	PA2210		
PA14_36345	<i>exoY</i>	adenylate cyclase	5,4	2,2E-32	PA2191	<i>exoY</i>	X
PA14_36960		putative transporter	6,8	8,8E-17	PA2135		
PA14_36980		hypothetical protein	9,7	3,0E-52	PA2134		
PA14_39850		putative MFS transporter	4,6	1,5E-13	PA1908		
PA14_42100		putative membrane transport protein	4,2	3,0E-12	PA1735		X
PA14_42290	<i>pscH</i>	type III export protein PscH	4,9	5,2E-16	PA1721	<i>pscH</i>	
PA14_42300	<i>pscG</i>	type III export protein PscG	4,9	9,8E-21	PA1720	<i>pscG</i>	X
PA14_42310	<i>pscF</i>	type III export protein PscF	6,1	2,0E-19	PA1719	<i>pscF</i>	
PA14_42320	<i>pscE</i>	type III export protein PscE	6,5	8,3E-25	PA1718	<i>pscE</i>	
PA14_42340	<i>pscD</i>	type III export protein PscD	6,7	1,2E-33	PA1717	<i>pscD</i>	
PA14_42350	<i>pscC</i>	Type III secretion outer membrane protein PscC precursor	8,9	8,0E-48	PA1716	<i>pscC</i>	
PA14_42360	<i>pscB</i>	type III export apparatus protein	15,2	6,8E-65	PA1715	<i>pscB</i>	X
PA14_42380		hypothetical protein	16,9	1,1E-81	PA1714	<i>exsD</i>	
PA14_42390	<i>exsA</i>	transcriptional regulator ExsA	4,1	4,8E-23	PA1713	<i>exsA</i>	
PA14_42400	<i>exsB</i>	exoenzyme S synthesis protein B	12,7	8,4E-66	PA1712	<i>exsB</i>	X
PA14_42410		hypothetical protein	11,5	4,3E-63	PA1711	<i>exsE</i>	X
PA14_42430	<i>exsC</i>	exoenzyme S synthesis protein C	14,3	1,4E-74	PA1710	<i>exsC</i>	X
PA14_42440	<i>popD</i>	translocator outer membrane protein PopD precursor	9,8	1,1E-57	PA1709	<i>popD</i>	X
PA14_42450	<i>popB</i>	translocator protein PopB	10,9	1,2E-62	PA1708	<i>popB</i>	X
PA14_42460	<i>pcrH</i>	regulatory protein PcrH	17,5	2,5E-82	PA1707	<i>pcrH</i>	X
PA14_42470	<i>pcrV</i>	type III secretion protein PcrV	26,0	3,9E-103	PA1706	<i>pcrV</i>	X
PA14_42480	<i>pcrG</i>	regulator in type III secretion	30,7	9,7E-100	PA1705	<i>pcrG</i>	X
PA14_42530	<i>pcr2</i>	type III secretion protein	6,3	3,1E-18	PA1700	<i>pcr2</i>	
PA14_42540	<i>pcr1</i>	protein in type III secretion	6,5	8,4E-18	PA1699	<i>pcr1</i>	
PA14_42570	<i>pscN</i>	type III secretion system ATPase	32,6	1,5E-74	PA1697		X
PA14_42610	<i>pscQ</i>	type III secretion system protein	18,8	6,4E-47	PA1694	<i>pscQ</i>	
PA14_43160		putative transporter	13,7	7,7E-54	PA1651		X
PA14_43170		bile acid/Na ⁺ symporter family transporter	12,1	9,5E-61	PA1650		X
PA14_44800		putative transporter	13,3	4,1E-47	PA1519		
PA14_46110		sodium:solute symport protein	5,9	3,9E-18	PA1418		X
PA14_46640		putative siderophore receptor	8,9	3,9E-18	PA1365		
PA14_46910		ABC transporter substrate-binding protein	5,9	1,3E-36	PA1342	<i>aatJ</i>	X
PA14_50770		putative transporter	22,3	1,4E-70	PA1051		X
PA14_51520	<i>spcU</i>	SpcU	9,1	4,6E-39	NA		
PA14_51530	<i>exoU</i>	ExoU	17,0	7,0E-85	NA		
PA14_51880	<i>oprD</i>	outer membrane porin OprD precursor	5,0	8,6E-31	PA0958	<i>oprD</i>	
PA14_54150	<i>putP</i>	sodium/proline symporter PutP	4,1	1,8E-21	PA0783	<i>putP</i>	
PA14_58350		ABC transporter substrate-binding protein	4,5	4,9E-26	PA4496	<i>dppA1</i>	
PA14_58390		ABC transporter substrate-binding protein	8,9	3,5E-53	PA4500	<i>dppA3</i>	X
PA14_58410		putative porin	20,7	1,8E-91	PA4501	<i>opdD</i>	
PA14_58420		dipeptide ABC transporter substrate-binding protein	4,9	5,4E-29	PA4502	<i>dppA4</i>	
PA14_58440		probable dipeptide ABC transporter permease	4,5	3,8E-25	PA4503	<i>dppB</i>	
PA14_64710		putative extracellular heme-binding protein	10,6	2,7E-54	PA4897		
PA14_64860	<i>livF</i>	ABC transporter ATP-binding protein	4,7	6,1E-27	PA4909		X
PA14_64870	<i>braF</i>	ABC transporter ATP-binding protein	8,7	9,8E-48	PA4910		X
PA14_64880	<i>braE</i>	branched chain amino acid ABC transporter permease	22,4	2,2E-84	PA4911		X
PA14_64890	<i>braD</i>	branched chain amino acid ABC transporter permease	24,6	5,1E-92	PA4912		X
PA14_64900		ABC transporter substrate-binding protein	14,6	4,0E-76	PA4913		X
PA14_67050		ABC transporter substrate-binding protein	5,7	4,5E-27	PA5076		
PA14_67130		ABC transporter substrate-binding protein	5,3	2,7E-24	PA5082	<i>dguC</i>	X
PA14_68060		ABC transporter ATP-binding protein	9,0	1,4E-48	PA5152		X
PA14_68070		putative periplasmic binding protein	18,4	8,4E-87	PA5153		X
PA14_68080		ABC transporter permease	11,2	3,2E-46	PA5154		X
PA14_68090		ABC transporter permease	5,8	5,2E-27	PA5155		
PA14_68260		c4-dicarboxylate-binding protein	5,0	1,1E-29	PA5167	<i>dctP</i>	X
PA14_68280		dicarboxylate transporter	8,8	9,6E-43	PA5168	<i>dctQ</i>	X
PA14_68290		C4-dicarboxylate transporter	8,3	8,2E-45	PA5169	<i>dctM</i>	X
PA14_69130	<i>glpT</i>	sn-glycerol-3-phosphate transporter	4,4	7,9E-22	PA5235	<i>glpT</i>	
PA14_70160		omega amino acid--pyruvate transaminase	5,6	4,6E-32	PA5313	<i>gabT2</i>	X
PA14_70850	<i>pstC</i>	membrane protein component of ABC phosphate transporter	5,7	1,7E-26	PA5368	<i>pstC</i>	
PA14_70860		putative transporter	10,7	2,3E-55	PA5369	<i>pstS</i>	
PA14_73100		hypothetical protein	5,7	3,5E-23	PA5543		
PA14_73110		putative transporter	17,9	6,6E-81	PA5544		
PA14_73120		putative transporter	15,4	4,3E-78	PA5544		
Energy metabolism							
PA14_01290	<i>coxB</i>	cytochrome c oxidase subunit II	28,3	8,8E-111	PA0105	<i>coxB</i>	
PA14_01300	<i>coxA</i>	cytochrome c oxidase subunit I	64,1	2,8E-155	PA0106	<i>coxA</i>	X
PA14_01310	<i>coxG</i>	cytochrome C oxidase assembly protein	71,1	1,5E-156	PA0107		X
PA14_01320	<i>coIII</i>	cytochrome c oxidase subunit III	28,7	1,4E-111	PA0108	<i>coIII</i>	X
PA14_01340		hypothetical protein	28,1	1,9E-106	PA0110		X
PA14_01350		hypothetical protein	19,7	4,2E-87	PA0111		X
PA14_01360		hypothetical protein	46,6	1,3E-130	PA0112		X
PA14_01380		protoheme IX farnesyltransferase	52,0	1,1E-139	PA0113		X
PA14_01390		hypothetical protein	9,4	1,4E-53	PA0114	<i>senC</i>	X
PA14_18060		putative cytochrome	5,4	3,8E-08	PA3575		
PA14_24860	<i>snr1</i>	cytochrome c Snr1	6,4	7,7E-38	PA3032	<i>snr1</i>	X
PA14_29050		molybdopterin oxidoreductase	4,3	8,8E-17	PA2714		
PA14_49250	<i>napA</i>	nitrate reductase catalytic subunit	4,3	6,4E-26	PA1174	<i>napA</i>	X
PA14_49260	<i>napB</i>	cytochrome c-type protein NapB precursor	6,9	3,2E-20	PA1173	<i>napB</i>	

PA14_49270	<i>napC</i>	cytochrome c-type protein NapC	13,6	2,5E-67	PA1172	<i>napC</i>	X
PA14_52380		cytochrome b561	7,5	5,4E-40	PA0918		
PA14_57560		cytochrome b	6,1	6,3E-34	PA4430		
PA14_63605	<i>fdnG</i>	formate dehydrogenase-O, major subunit	6,3	4,6E-39	PA4812	<i>fdnG</i>	
Virulence and other functions							
PA14_05690	<i>codA</i>	cytosine deaminase	21,5	8,0E-76	PA0437	<i>codA</i>	
PA14_05700	<i>codB</i>	cytosine permease	68,3	2,3E-110	PA0438	<i>codB</i>	
PA14_05740	<i>pydA</i>	dihydropyrimidine dehydrogenase	2753,2	0,0E+00	PA0439		
PA14_05750		oxidoreductase	1742,6	0,0E+00	PA0440		
PA14_05770	<i>dht</i>	phenylhydantoinease	926,9	0,0E+00	PA0441	<i>dht</i>	X
PA14_05810	<i>amaB</i>	allantoate amidohydrolase	225,7	8,8E-220	PA0444		
PA14_06500	<i>bioB</i>	biotin synthase	5,3	1,7E-31	PA0500	<i>bioB</i>	
PA14_09490	<i>phzM</i>	phenazine-specific methyltransferase	9,8	1,1E-57	PA4209	<i>phzM</i>	
PA14_09980	<i>dkgB</i>	2,5-diketo-D-gluconate reductase B	4,9	2,1E-14	PA4167		
PA14_14740		scaffold protein	5,2	1,6E-32	PA3813	<i>iscU</i>	X
PA14_14750		iron-binding protein	4,4	5,8E-27	PA3812	<i>iscA</i>	X
PA14_14770	<i>hscB</i>	co-chaperone HscB	4,2	2,2E-25	PA3811	<i>hscB</i>	X
PA14_20140	<i>fpr</i>	ferredoxin--NADP+ reductase	11,9	9,7E-64	PA3397	<i>fprA</i>	
PA14_23680	<i>ibpA</i>	heat-shock protein IbpA	16,3	1,0E-70	PA3126	<i>ibpA</i>	X
PA14_23770		putative biotin synthesis protein	6,3	6,0E-29	PA3119		
PA14_24650	<i>rmf</i>	ribosome modulation factor	9,7	2,3E-58	PA3049	<i>rmf</i>	
PA14_27100	<i>lipA</i>	lactonizing lipase	4,9	4,4E-15	PA2862	<i>lipA</i>	X
PA14_27220	<i>ohr</i>	organic hydroperoxide resistance protein	100,1	5,7E-175	PA2850	<i>ohr</i>	
PA14_27480	<i>htpX</i>	heat shock protein HtpX	5,4	1,0E-31	PA2830	<i>htpX</i>	
PA14_28580		hypothetical protein	6,4	2,9E-20	NA		
PA14_28590		methionine aminopeptidase	5,6	6,2E-25	PA2748		
PA14_28970		hypothetical protein	13,0	6,6E-56	NA		
PA14_28980		Fe2+-dicitrate sensor	10,3	2,5E-40	NA		
PA14_29320		NADH dehydrogenase, FAD-containing subunit	74,5	1,8E-162	PA2691		X
PA14_33590		hypothetical protein	4,4	8,9E-24	PA2404	<i>fpvH</i>	X
PA14_33690	<i>pvdE</i>	pyoverdine biosynthesis protein PvdE	6,3	3,1E-36	PA2397	<i>pvdE</i>	
PA14_33700	<i>pvdF</i>	pyoverdine synthetase F	6,3	2,2E-09	PA2396	<i>pvdF</i>	X
PA14_36290		hypothetical protein	4,0	6,9E-21	PA2197		
PA14_36810	<i>katE</i>	hydroperoxidase II	16,1	8,1E-60	PA2147	<i>katE</i>	
PA14_36820		hypothetical protein	6,7	1,3E-40	PA2146		X
PA14_36910	<i>ligD</i>	ATP-dependent DNA ligase	4,3	4,4E-19	PA2138	<i>ligD</i>	X
PA14_44560		flavin-containing monooxygenase	5,4	5,7E-20	PA1538		
PA14_45100		putative mucoidy inhibitor gene A	10,4	5,4E-45	PA1494	<i>muiA</i>	
PA14_49410		cold-shock protein	4,2	4,6E-17	PA1159		
PA14_49460	<i>nrpA</i>	ribonucleotide-diphosphate reductase subunit alpha	4,6	1,7E-27	PA1156	<i>nrpA</i>	
PA14_52480		putative autolysis protein	8,8	3,2E-41	PA0911	<i>alpE</i>	
PA14_52490		putative autolysis protein	13,2	1,1E-54	PA0910	<i>alpD</i>	X
PA14_52510		putative autolysis protein	6,3	9,7E-20	PA0908	<i>alpB</i>	
PA14_53820		putative β -lactamase expression regulator	6,5	4,3E-39	PA0807	<i>ampDh3</i>	X
PA14_54620		aldehyde dehydrogenase	4,1	1,2E-23	PA0747		
PA14_54630		acyl-CoA dehydrogenase	4,4	5,1E-26	PA0746		X
PA14_56780	<i>sodB</i>	superoxide dismutase	8,5	4,3E-50	PA4366	<i>sodB</i>	
PA14_64840		short-chain dehydrogenase	4,2	4,4E-19	PA4907		X
PA14_65740	<i>thiC</i>	thiamine biosynthesis protein ThiC	16,3	1,0E-80	PA4973	<i>thiC</i>	
PA14_66770	<i>hslV</i>	ATP-dependent protease peptidase subunit	4,6	2,5E-15	PA5053	<i>hslV</i>	
PA14_70600	<i>hupA</i>	HU family DNA-binding protein	42,0	1,4E-131	PA5348		X
PA14_72870		aminotransferase	4,0	1,6E-18	PA5523		X
Hypothetical proteins, unknown function							
PA14_00130		hypothetical protein	5,2	6,7E-09	PA0012		
PA14_02970		hypothetical protein	5,0	1,1E-08	PA0239		
PA14_01170		hypothetical protein	5,2	3,0E-20	PA0096		
PA14_01180		hypothetical protein	4,7	5,4E-21	PA0097		
PA14_01540		hypothetical protein	5,6	2,0E-22	PA0126		
PA14_03560		hypothetical protein	23,8	2,5E-58	PA0274		X
PA14_07330		hypothetical protein	4,0	5,4E-19	PA0563		
PA14_07370		hypothetical protein	5,0	4,6E-29	PA0567		X
PA14_13130		hypothetical protein	5,2	2,9E-31	PA3923		X
PA14_14540		hypothetical protein	21,3	1,4E-30	NA		
PA14_14550		hypothetical protein	11,2	3,8E-44	NA		
PA14_18810		hypothetical protein	8,8	3,5E-29	PA3519		
PA14_18870		hypothetical protein	4,4	8,4E-24	PA3496		
PA14_19170		hypothetical protein	5,2	5,2E-17	PA3472		
PA14_19680		hypothetical protein	10,8	5,6E-47	PA3432		X
PA14_19690		hypothetical protein	5,6	4,4E-31	PA3431		X
PA14_20460		hypothetical protein	5,8	2,7E-32	PA3371		X
PA14_20470		hypothetical protein	5,9	2,0E-33	NA		
PA14_24990		hypothetical protein	4,0	9,2E-20	PA3021		
PA14_26060		hypothetical protein	4,6	8,3E-27	PA2937		X
PA14_28600		hypothetical protein	4,1	1,2E-23	PA2747		X
PA14_30410		hypothetical protein	12,3	1,8E-68	PA2604		
PA14_36170		hypothetical protein	6,3	8,2E-11	PA2207		
PA14_36375		hypothetical protein	4,5	4,3E-20	PA2180		X
PA14_36410		hypothetical protein	4,3	2,1E-18	PA2178		
PA14_36490		hypothetical protein	6,2	1,6E-33	PA2173		X
PA14_36500		hypothetical protein	9,8	4,5E-47	PA2172		X
PA14_36520		hypothetical protein	17,1	2,9E-80	PA2171		X
PA14_36530		hypothetical protein	6,4	1,4E-37	PA2169		
PA14_36560		hypothetical protein	18,9	2,1E-87	PA2166		X
PA14_36650		hypothetical protein	4,9	3,4E-29	PA2159		X
PA14_36740		hypothetical protein	4,5	1,2E-24	PA2151		X
PA14_38610		hypothetical protein	25,7	7,0E-102	PA2002		
PA14_39440		hypothetical protein	8,5	7,6E-44	PA1941		
PA14_39830		hypothetical protein	4,3	4,8E-15	PA1909		
PA14_39990		hypothetical protein	734,3	4,4E-265	PA1897		
PA14_40010		hypothetical protein	304,4	3,2E-225	PA1896		
PA14_40020		hypothetical protein	142,2	2,6E-193	PA1895		
PA14_40030		hypothetical protein	104,5	1,1E-174	PA1894		

PA14_40040	hypothetical protein	63,6	9,8E-148	PA1893	
PA14_40050	hypothetical protein	51,0	4,3E-125	PA1892	
PA14_40060	hypothetical protein	51,8	6,4E-44	PA1891	
PA14_40560	hypothetical protein	4,9	2,2E-28	PA1852	X
PA14_40750	hypothetical protein	4,1	2,0E-24	NA	
PA14_41750	hypothetical protein	9,3	1,5E-41	PA1764	
PA14_41760	hypothetical protein	5,9	3,4E-32	PA1763	X
PA14_41790	hypothetical protein	13,6	6,9E-72	PA1761	X
PA14_44170	hypothetical protein	4,6	8,2E-26	PA1571	
PA14_44510	hypothetical protein	6,2	2,2E-26	PA1542	
PA14_46280	hypothetical protein	10,6	1,3E-57	PA1404	
PA14_46770	hypothetical protein	8,0	2,7E-38	PA1354	
PA14_46780	hypothetical protein	6,2	8,6E-31	PA1353	
PA14_47010	hypothetical protein	4,6	6,0E-24	PA1333	
PA14_49050	hypothetical protein	56,3	8,1E-147	PA1190	X
PA14_49480	hypothetical protein	6,2	1,1E-05	NA	
PA14_49650	hypothetical protein	4,1	4,0E-19	PA1140	X
PA14_50940	hypothetical protein	4,1	1,6E-21	PA1035	X
PA14_51590	hypothetical protein	5,0	7,5E-08	NA	
PA14_53390	hypothetical protein	11,4	3,1E-47	PA0841	
PA14_53620	hypothetical protein	28,6	1,9E-30	PA0826	X
PA14_53840	hypothetical protein	4,8	3,3E-15	PA0805	X
PA14_54870	hypothetical protein	11,4	1,0E-62	NA	
PA14_56090	hypothetical protein	4,0	1,2E-16	PA4317	
PA14_59550	hypothetical protein	6,7	7,8E-08	NA	
PA14_60480	hypothetical protein	13,2	1,4E-71	PA4570	
PA14_60630	hypothetical protein	4,1	4,7E-20	PA4582	
PA14_61270	hypothetical protein	5,4	1,3E-27	PA4630	X
PA14_65920	hypothetical protein	21,8	2,9E-66	PA4985	
PA14_71900	hypothetical protein	11,4	5,5E-66	PA5446	
PA14_72060	hypothetical protein	35,9	1,6E-111	PA5460	X
PA14_72360	hypothetical protein	5,6	2,3E-34	PA5481	X
PA14_72660	hypothetical protein	4,2	2,5E-13	PA5507	
PA14_72930	hypothetical protein	9,2	8,6E-53	PA5528	
a Gene numbers and functions are taken from the Pseudomonas genome database (Winsor et al., 2016)					
b Operons in PA14, which are boxed, and gene homologs in PAO1 were taken from Wurtzel et al., 2012					
c c-di-GMP regulated genes were taken from Lin Chua et al., 2017					

Supplementary Table S3. Transcripts with decreased abundance in PA14Δ*hly* versus PA14

PA14 id ^{a,b}	Gene	Description ^a	foldChange	padj	Gene homologs in PAO1 ^{a,1}	Gene PAO1	c-di-GMP dep. ^c
Carbon and amino acid metabolism							
PA14_02730		dihydrodipicolinate synthetase	-5,1	2,4E-09	PA0223		
PA14_06960		hypothetical protein	-15,1	6,4E-68	PA0534	<i>pauB1</i>	
PA14_06970		Cro/CI family transcriptional regulator	-8,3	9,3E-30	PA0535		X
PA14_09210	<i>pchA</i>	salicylate biosynthesis isochorismate synthase	-6,7	1,2E-43	PA4231	<i>pchA</i>	
PA14_09220	<i>pchB</i>	isochorismate-pyruvate lyase	-4,8	3,6E-30	PA4230	<i>pchB</i>	
PA14_09240	<i>pchD</i>	pyochelin biosynthesis protein PchD	-4,5	2,3E-04	PA4228	<i>pchD</i>	
PA14_10550	<i>cysI</i>	sulfite reductase	-19,5	2,0E-93	PA4130		
PA14_10560		hypothetical protein	-26,8	3,6E-105	PA4129		
PA14_19590	<i>ssuF</i>	molybdopterin-binding protein	-8,1	1,8E-10	PA3441		X
PA14_34870	<i>chiC</i>	chitinase	-18,3	9,1E-91	PA2300	<i>chiC</i>	X
PA14_38510	<i>hmgA</i>	homogentisate 1,2-dioxygenase	-20,0	3,6E-91	PA2009	<i>hmgA</i>	X
PA14_38530	<i>fahA</i>	fumarylacetoacetase	-21,5	1,7E-95	PA2008	<i>fahA</i>	X
PA14_38550	<i>maiA</i>	maleylacetoacetate isomerase	-27,8	4,1E-29	PA2007	<i>maiA</i>	X
PA14_48570		2-isopropylmalate synthase	-12,2	2,7E-48	PA1217		
PA14_48590		hypothetical protein	-10,1	9,6E-41	PA1216		
PA14_48600		AMP-binding protein	-16,0	2,2E-47	PA1215		
PA14_48610		asparagine synthase	-13,8	5,9E-34	PA1214		X
PA14_48620		clavaminic acid synthetase	-9,0	8,1E-22	PA1213		
PA14_53010	<i>phhC</i>	aromatic amino acid aminotransferase	-5,8	3,4E-37	PA0870	<i>phhC</i>	X
PA14_53450		hypothetical protein	-4,7	6,3E-04	NA		
PA14_53470	<i>ackA</i>	acetate kinase	-5,5	4,0E-34	PA0836	<i>ackA</i>	
PA14_53480	<i>pta</i>	phosphate acetyltransferase	-4,5	1,3E-06	PA0835	<i>pta</i>	
PA14_54000	<i>prpD</i>	2-methylcitrate dehydratase	-5,0	2,6E-31	PA0792	<i>prpD</i>	X
PA14_66440	<i>metY</i>	O-acetylhomoserine aminocarboxypropyltransferase	-6,9	9,8E-05	PA5025	<i>metY</i>	
PA14_68300	<i>arcD</i>	arginine/ornithine antiporter	-5,2	1,0E-33	PA5170	<i>arcD</i>	X
PA14_71630	<i>adhA</i>	alcohol dehydrogenase	-26,8	9,3E-67	PA5427	<i>adhA</i>	X
Fatty acid metabolism and cell wall							
PA14_05840	<i>gcdH</i>	glutaryl-CoA dehydrogenase	-10,6	2,7E-09	PA0447	<i>gcdH</i>	
PA14_12450		acyl-CoA dehydrogenase	-5,3	1,4E-32	PA3972		
PA14_12470		hypothetical protein	-5,4	1,2E-13	PA3971		
PA14_20890	<i>rfaD</i>	ADP-L-glycero-D-manno-heptose-6-epimerase	-237,9	6,6E-202	PA3337	<i>rfaD</i>	X
PA14_23410	<i>orfJ</i>	glycosyl transferase family protein	-5,7	1,1E-13	NA		
PA14_23450	<i>orfM</i>	NAD dependent epimerase/dehydratase	-6,7	1,3E-13	PA3146	<i>whpK</i>	
PA14_23460	<i>orfN</i>	group 4 glycosyl transferase	-6,2	1,2E-18	PA3145	<i>whpL</i>	
PA14_25660	<i>fabG</i>	3-ketoacyl-ACP reductase	-4,5	5,0E-27	NA		
PA14_26690		enoyl-CoA hydratase/isomerase	-5,0	1,0E-11	PA2890	<i>atuE</i>	
PA14_31530		acyl-CoA thiolase	-4,8	3,0E-30	PA2553		X
PA14_31540	<i>acdA</i>	acyl-CoA dehydrogenase	-4,5	2,7E-28	PA2552		X
PA14_31580		acyl-CoA dehydrogenase	-7,3	4,1E-10	PA2550		X
PA14_56570		acyltransferase	-26,8	1,4E-41	PA4351	<i>olsA</i>	X
PA14_66350		acyl-CoA dehydrogenase	-7,1	8,4E-11	PA5020		X
Regulators							
PA14_06860		hypothetical protein	-31,7	7,1E-09	PA0526		
PA14_06870	<i>dnr</i>	transcriptional regulator Dnr	-5,2	5,0E-02	PA0527	<i>dnr</i>	
PA14_10530		GntR family transcriptional regulator	-11,3	1,4E-19	PA4132		
PA14_10540		iron-sulfur cluster-binding protein	-11,1	2,0E-59	PA4131		
PA14_11120	<i>rcsB</i>	response regulator	-5,5	1,8E-23	PA4080		
PA14_16280		putative transcriptional regulator	-11,0	7,2E-05	PA3721	<i>nalC</i>	
PA14_16290		hypothetical protein	-5,7	2,7E-25	PA3720		
PA14_16300		putative antirepressor for MexR	-8,3	4,2E-22	PA3719	<i>armR</i>	
PA14_16790		TetR family transcriptional regulator	-6,0	5,5E-30	PA3678	<i>mexL</i>	X
PA14_19380		putative transcriptional regulator	-9,7	1,5E-52	PA3458		
PA14_25180	<i>psrA</i>	transcriptional regulator PsrA	-4,2	4,2E-02	PA3006	<i>psrA</i>	X
PA14_34660	<i>gntR</i>	transcriptional regulator GntR	-9,6	3,3E-42	PA2320	<i>gntR</i>	
PA14_34880		GntR family transcriptional regulator	-5,1	9,2E-21	PA2299		X
PA14_37070		putative cupA gene regulator A	-23,6	2,1E-21	PA2127	<i>cgrA</i>	
PA14_37080		putative cupA gene regulator C	-7,8	1,6E-23	PA2126	<i>cgrC</i>	
PA14_38500		IclR family transcriptional regulator	-23,3	1,2E-34	PA2010		X
PA14_38570		putative transcriptional regulator	-5,1	7,1E-11	PA2005	<i>hbcR</i>	
PA14_48810		NAD-dependent deacetylase	-8,4	1,4E-42	PA1197		
PA14_48830		putative transcriptional regulator	-22,4	3,6E-87	PA1196	<i>ddaR</i>	X
PA14_59780	<i>rcsC</i>	kinase sensor protein	-5,5	4,4E-33	NA		
PA14_59790	<i>pvrR</i>	two component response regulator	-10,5	1,1E-05	NA		
PA14_64930		putative nudix-related transcriptional regulator	-4,9	3,2E-29	PA4916	<i>ntrR</i>	
PA14_67680	<i>ntrC</i>	two-component response regulator NtrC	-5,9	1,4E-32	PA5125	<i>ntrC</i>	
Transporters, porins, efflux pumps and secretion							
PA14_09270	<i>pchE</i>	dihydroaeruginic acid synthetase	-10,4	2,0E-63	PA4226	<i>pchE</i>	
PA14_09280	<i>pchF</i>	pyochelin synthetase	-13,9	8,2E-46	PA4225	<i>pchF</i>	
PA14_09290	<i>pchG</i>	pyochelin biosynthetic protein PchG	-11,1	7,5E-34	PA4224	<i>pchG</i>	
PA14_09300		ABC transporter ATP-binding protein	-10,6	7,2E-63	PA4223		
PA14_09320		ABC transporter ATP-binding protein	-10,3	3,7E-38	PA4221	<i>fpfA</i>	
PA14_09380		transporter	-4,1	3,2E-24	PA4218	<i>ampP</i>	
PA14_09500	<i>opmD</i>	outer membrane protein	-10,2	9,3E-43	PA4208	<i>opmD</i>	X
PA14_11270	<i>oprG</i>	outer membrane protein OprG precursor	-26,0	9,3E-110	PA4067	<i>oprG</i>	X
PA14_13170		metal transporting P-type ATPase	-5,1	8,2E-26	PA3920		
PA14_14360		sodium:sulfate symporter	-7,3	3,5E-40	PA3839		
PA14_19580	<i>ssuB</i>	aliphatic sulfonates transport ATP-binding subunit	-8,6	2,8E-09	PA3442		
PA14_20030	<i>hasD</i>	transport protein HasD	-4,8	1,0E-20	PA3406	<i>hasD</i>	X
PA14_20040	<i>hasE</i>	metalloprotease secretion protein	-5,0	8,8E-27	PA3405	<i>hasE</i>	X
PA14_20050		outer membrane protein	-6,8	4,1E-24	PA3404		X
PA14_22980		sugar ABC transporter substrate-binding protein	-22,8	3,2E-98	PA3190		X
PA14_22990		ABC sugar transporter permease	-15,0	5,2E-39	PA3189		X
PA14_23000		ABC sugar transporter permease	-21,3	1,5E-26	PA3188		X
PA14_23010	<i>gltK</i>	ABC transporter ATP-binding protein	-52,8	1,8E-122	PA3187		X
PA14_23030	<i>oprB</i>	glucose/carbohydrate outer membrane porin OprB precursor	-20,3	8,1E-77	PA3186	<i>oprB</i>	X
PA14_34490		hypothetical protein	-16,7	1,9E-59	PA2330		
PA14_34500		ABC transporter ATP-binding protein	-11,8	5,5E-19	PA2329		

PA14_34510		hypothetical protein	-15,2	2,1E-33	PA2328		
PA14_34520		ABC transporter permease	-9,0	5,5E-22	PA2327		
PA14_38560	<i>pcaK</i>	MFS transporter	-19,3	4,2E-22	PA2006		X
PA14_42890	<i>stp1</i>	serine/threonine phosphoprotein phosphatase Stp1	-4,5	4,8E-14	PA1670	<i>stp1</i>	
PA14_42940		lipoprotein	-7,6	1,1E-20	PA1666	<i>lip2</i>	
PA14_42950		putative type VI secretion system protein	-9,9	4,9E-34	PA1665	<i>fha2</i>	
PA14_42970		putative transcriptional regulator	-9,0	1,0E-28	PA1663	<i>sfa2</i>	X
PA14_42980		putative ClpA/B-type protease	-6,0	4,4E-28	PA1662	<i>clpV2</i>	
PA14_42990		putative type VI secretion system protein	-8,9	2,1E-14	PA1661	<i>hsiH2</i>	
PA14_43000		putative type VI secretion system protein	-8,4	1,3E-33	PA1660	<i>hsiG2</i>	
PA14_43020		putative type VI secretion system protein	-8,1	4,0E-10	PA1659	<i>hsiF2</i>	
PA14_43030		putative type VI secretion system protein	-9,8	6,5E-27	PA1658	<i>hsiC2</i>	
PA14_43040		putative type VI secretion system protein	-9,5	4,5E-44	PA1657	<i>hsiB2</i>	X
PA14_43050		putative type VI secretion system protein	-24,5	4,9E-99	PA1656	<i>hsiA2</i>	X
PA14_43070	<i>hcp2</i>	secreted protein Hcp	-16,7	8,4E-37	NA		
PA14_45970		cation-transporting P-type ATPase	-29,9	2,9E-91	PA1429		X
PA14_48100	<i>aprE</i>	alkaline protease secretion protein AprE	-4,2	5,5E-15	PA1247	<i>aprE</i>	X
PA14_48115	<i>aprD</i>	alkaline protease secretion protein AprD	-4,1	4,8E-11	PA1246	<i>aprD</i>	X
PA14_49130	<i>dctA</i>	C4-dicarboxylate transporter DctA	-8,9	3,3E-07	PA1183	<i>dctA</i>	
PA14_56670		hypothetical protein	-424,7	2,5E-164	PA4357		
PA14_56680	<i>feoB</i>	ferrous iron transport protein B	-119,1	9,8E-50	PA4358	<i>feoB</i>	
PA14_56690	<i>feoA</i>	ferrous iron transport protein A	-220,4	1,0E-24	PA4359		X
PA14_69060		ABC transporter permease	-5,2	7,0E-16	PA5230		
PA14_69070		ABC transporter ATP-binding protein/permease	-17,7	2,2E-84	PA5231		X
PA14_69090		hypothetical protein	-27,2	9,4E-27	PA5232		X
Energy metabolism							
PA14_06750	<i>nirS</i>	nitrite reductase	-12,1	1,1E-28	PA0519	<i>nirS</i>	X
PA14_10490		hypothetical protein	-26,4	1,6E-54	PA4134		
PA14_10500		cbb3-type cytochrome c oxidase subunit I	-17,9	7,7E-33	PA4133		
PA14_13730	<i>narL</i>	transcriptional regulator NarL	-5,5	2,4E-30	PA3879	<i>narL</i>	
PA14_13750	<i>narK1</i>	nitrite extrusion protein 1	-44,2	8,1E-68	PA3877	<i>narK1</i>	
PA14_13770	<i>narK2</i>	nitrite extrusion protein 2	-12,7	5,2E-18	PA3876	<i>narK2</i>	
PA14_13780	<i>narG</i>	respiratory nitrate reductase alpha subun	-4,3	1,5E-11	PA3875	<i>narG</i>	
PA14_29940	<i>nuoG</i>	NADH dehydrogenase subunit G	-4,0	2,2E-23	PA2642	<i>nuoG</i>	
PA14_44340		cbb3-type cytochrome c oxidase subunit I	-7,0	2,1E-17	PA1557	<i>ccoN2</i>	
PA14_44350		cbb3-type cytochrome c oxidase subunit II	-35,9	1,1E-87	PA1556	<i>ccoO2</i>	
PA14_44360		cytochrome c oxidase, cbb3-type subunit III	-11,9	9,9E-57	PA1555	<i>ccoP2</i>	
PA14_44470	<i>hemN</i>	coproporphyrinogen III oxidase	-89,0	6,9E-31	PA1546	<i>hemN</i>	
PA14_47150	<i>cyoE</i>	protoheme IX farnesyltransferase	-6,8	7,7E-09	PA1321	<i>cyoE</i>	
PA14_47160	<i>cyoD</i>	cytochrome o ubiquinol oxidase subunit IV	-10,7	1,4E-08	PA1320	<i>cyoD</i>	X
PA14_47180	<i>cyoC</i>	cytochrome o ubiquinol oxidase subunit III	-13,3	2,5E-20	PA1319	<i>cyoC</i>	X
PA14_47190	<i>cyoB</i>	cytochrome o ubiquinol oxidase subunit I	-12,1	3,9E-51	PA1318	<i>cyoB</i>	
PA14_47210	<i>cyoA</i>	cytochrome o ubiquinol oxidase subunit II	-16,5	3,3E-21	PA1317	<i>cyoA</i>	X
PA14_60490		cytochrome c	-4,8	2,0E-09	PA4571		
PA14_60700	<i>ccpR</i>	cytochrome c551 peroxidase	-57,2	1,9E-28	PA4587	<i>ccpR</i>	X
Virulence and other functions							
PA14_06000	<i>clpA</i>	ClpA/B protease ATP binding subunit	-8,5	2,3E-53	PA0459		
PA14_08750	<i>rplL</i>	50S ribosomal protein L7/L12	-4,1	1,5E-07	PA4271	<i>rplL</i>	X
PA14_08780	<i>rpoC</i>	DNA-directed RNA polymerase subunit beta'	-5,1	2,3E-33	PA4269	<i>rpoC</i>	X
PA14_09400	<i>phzS</i>	hypothetical protein	-5,8	7,0E-38	PA4217	<i>phzS</i>	
PA14_09440	<i>phzE1</i>	phenazine biosynthesis protein PhzE	-5,5	8,2E-11	PA4214	<i>phzE1</i>	X
PA14_09900	<i>prpL</i>	PvdS-regulated endoprotease, lysyl class	-4,3	4,3E-11	PA4175	<i>piv</i>	X
PA14_09950		oxidoreductase	-5,3	2,6E-30	PA4170		
PA14_11130		short chain dehydrogenase	-4,4	8,2E-23	PA4079		
PA14_13340		extracellular nuclease	-4,9	2,7E-17	PA3909	<i>eddB</i>	
PA14_15530	<i>trbK</i>	entry/exclusion protein TrbK	-20,5	2,9E-10	NA		
PA14_19100	<i>rhlA</i>	rhamnosyltransferase chain A	-7,1	1,2E-11	PA3479	<i>rhlA</i>	X
PA14_19110	<i>rhlB</i>	rhamnosyltransferase chain B	-4,8	1,5E-29	PA3478	<i>rhlB</i>	X
PA14_19730		oxidoreductase	-4,6	2,4E-03	PA3427		X
PA14_21640		short chain dehydrogenase	-6,6	4,2E-25	PA3277		X
PA14_27000		chemotaxis transducer	-6,4	2,3E-09	PA2867		
PA14_29240		hydrolase	-7,1	6,2E-30	PA2698		
PA14_30630	<i>pqsH</i>	FAD-dependent monooxygenase	-4,7	5,3E-04	PA2587	<i>pqsH</i>	X
PA14_31290	<i>palL</i>	PA-I galactophilic lectin	-6,3	3,3E-39	PA2570	<i>lecA</i>	X
PA14_31510		short-chain dehydrogenase	-4,9	5,1E-31	PA2554		X
PA14_34540		xenobiotic compound DszA family monooxygenase	-6,7	3,6E-25	PA2326		
PA14_34840		non-ribosomal peptide synthetase	-4,5	4,1E-28	PA2302	<i>ambE</i>	X
PA14_39880	<i>phzG2</i>	pyridoxamine 5'-phosphate oxidase	-44,9	2,0E-96	PA1905	<i>phzG2</i>	X
PA14_39945	<i>phzC2</i>	phenazine biosynthesis protein PhzC	-10,7	4,9E-08	PA4212	<i>phzC1</i>	
PA14_39960	<i>phzB2</i>	phenazine biosynthesis protein	-5,2	1,7E-13	PA1900	<i>phzB2</i>	X
PA14_39970	<i>phzA2</i>	phenazine biosynthesis protein	-4,2	3,7E-15	PA1899	<i>phzA2</i>	X
PA14_44300	<i>aer</i>	aerotaxis receptor Aer	-9,5	3,0E-58	PA1561	<i>aer</i>	X
PA14_46890		short-chain dehydrogenase	-4,2	3,4E-23	PA1344		
PA14_48060	<i>aprA</i>	alkaline metalloproteinase	-23,5	3,0E-106	PA1249	<i>aprA</i>	X
PA14_48140		hypothetical protein	-10,0	2,5E-61	PA1245	<i>aprX</i>	X
PA14_49300		lipoygenase	-5,1	4,1E-25	PA1169		X
PA14_49310		hypothetical protein	-14,7	2,0E-45	PA1168		X
PA14_50800	<i>pdxH</i>	pyridoxamine 5'-phosphate oxidase	-4,4	6,9E-04	PA1049	<i>pdxH</i>	X
PA14_53400		oxidoreductase	-6,0	6,3E-06	PA0840		
PA14_56530		putative beta-lactamase	-4,3	4,1E-12	PA4347		
PA14_58230		putative protease inhibitor related	-4,8	6,7E-20	PA4487	<i>magF</i>	
PA14_60190	<i>clpB</i>	clpB protein	-5,4	1,8E-34	PA4542	<i>clpB</i>	X
PA14_66750	<i>argS</i>	arginyl-tRNA synthetase	-14,5	1,7E-68	PA5051	<i>argS</i>	
PA14_66760		hypothetical protein	-4,0	2,8E-13	PA5052		
Hypothetical proteins, unknown function							
PA14_01330		hypothetical protein	-5,1	8,5E-15	PA0109		X
PA14_02520		hypothetical protein	-58,5	2,9E-114	PA0200		X
PA14_05640		hypothetical protein	-4,6	5,4E-14	PA0434		X
PA14_05820		hypothetical protein	-9,0	1,2E-05	PA0446		
PA14_07430		hypothetical protein	-11,7	8,6E-67	PA0572		X
PA14_10360		hypothetical protein	-22,4	7,1E-104	PA4141		X

PA14_10370	hypothetical protein	-17,2	8,5E-46	PA4140	X
PA14_10380	hypothetical protein	-13,8	3,9E-07	PA4139	X
PA14_13720	hypothetical protein	-15,4	3,6E-21	PA3880	
PA14_14430	hypothetical protein	-5,0	1,1E-16	PA3835	
PA14_15050	hypothetical protein	-5,5	2,4E-08	PA3791	
PA14_15080	hypothetical protein	-4,6	6,3E-24	PA3789	X
PA14_15120	hypothetical protein	-4,0	2,6E-14	PA3785	X
PA14_16620	hypothetical protein	-4,6	1,4E-16	PA3693	
PA14_17570	hypothetical protein	-4,7	2,6E-29	PA3614	X
PA14_17580	hypothetical protein	-9,0	2,6E-11	PA3613	
PA14_21220	hypothetical protein	-13,1	2,3E-70	PA3309	
PA14_21260	hypothetical protein	-383,0	1,6E-44	NA	
PA14_21630	hypothetical protein	-7,8	9,5E-23	PA3278	X
PA14_28500	hypothetical protein	-81,2	2,1E-36	PA2753	X
PA14_28850	hypothetical protein	-4,2	2,5E-06	NA	
PA14_33300	hypothetical protein	-4,3	1,7E-12	NA	
PA14_33310	hypothetical protein	-4,2	1,3E-11	NA	
PA14_33360	hypothetical protein	-27,8	1,1E-05	NA	
PA14_34460	hypothetical protein	-29,2	1,2E-70	PA2331	
PA14_41970	hypothetical protein	-54,2	5,7E-103	PA1746	X
PA14_42860	hypothetical protein	-103,1	9,7E-178	PA1673	X
PA14_43760	hypothetical protein	-5,4	3,6E-06	PA1604	
PA14_48560	hypothetical protein	-12,4	7,1E-19	PA1218	
PA14_49320	hypothetical protein	-4,6	9,4E-06	PA1167	
PA14_49850	hypothetical protein	-11,1	2,7E-16	PA1123	
PA14_51570	hypothetical protein	-5,0	3,6E-07	NA	
PA14_54180	hypothetical protein	-8,2	3,0E-50	PA0781	
PA14_55110	hypothetical protein	-15,1	1,5E-03	PA0713	X
PA14_56170	hypothetical protein	-5,1	6,5E-15	PA4323	
PA14_56540	hypothetical protein	-80,9	1,1E-16	PA4348	X
PA14_56590	hypothetical protein	-72,8	1,5E-166	PA4352	X
PA14_56990	hypothetical protein	-5,0	8,2E-07	PA4384	
PA14_59970	hypothetical protein	-5,6	1,8E-04	NA	
PA14_60560	hypothetical protein	-13,6	3,1E-72	PA4577	
PA14_61000	hypothetical protein	-47,3	1,8E-13	PA4610	
PA14_65630	hypothetical protein	-7,0	3,9E-04	PA4965	
PA14_66460	hypothetical protein	-189,2	2,7E-192	PA5027	
PA14_67540	hypothetical protein	-6,0	6,4E-31	PA5115	
PA14_71380	hypothetical protein	-5,1	1,4E-25	PA5115	
PA14_72260	hypothetical protein	-284,3	7,2E-232	PA5475	X
PA14_72350	hypothetical protein	-5,6	1,5E-04	PA5480	
a Gene numbers and functions are taken from the Pseudomonas genome database (Winsor et al., 2016)					
b Operons in PA14, which are boxed, and gene homologs in PAO1 were taken from Wurtzel et al., 2012					
c c-di-GMP regulated genes were taken from Lin Chua et al., 2017					

5.4 Distinctive regulation of carbapenem susceptibility in *Pseudomonas aeruginosa* by Hfq



Distinctive Regulation of Carbapenem Susceptibility in *Pseudomonas aeruginosa* by Hfq

Elisabeth Sonleitner^{1*}, Petra Pusic¹, Michael T. Wolfinger^{2,3} and Udo Bläsi^{1*}

¹ Department of Microbiology, Immunobiology and Genetics, Max Perutz Labs, Vienna BioCenter (VBC), University of Vienna, Vienna, Austria, ² Department of Theoretical Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria, ³ Research Group Bioinformatics and Computational Biology, Faculty of Computer Science, University of Vienna, Vienna, Austria

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Denmark

*Correspondence:

Elisabeth Sonleitner
elisabeth.sonleitner@univie.ac.at
Udo Bläsi
udo.blaesi@univie.ac.at

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Carbapenems are often the antibiotics of choice to combat life threatening infections caused by the opportunistic human pathogen *Pseudomonas aeruginosa*. The outer membrane porins OprD and OpdP serve as entry ports for carbapenems. Here, we report that the RNA chaperone Hfq governs post-transcriptional regulation of the *oprD* and *opdP* genes in a distinctive manner. Hfq together with the recently described small regulatory RNAs (sRNAs) ErsA and Sr0161 is shown to mediate translational repression of *oprD*, whereas *opdP* appears not to be regulated by sRNAs. At variance, our data indicate that *opdP* is translationally repressed by a regulatory complex consisting of Hfq and the catabolite repression protein Crc, an assembly known to be key to carbon catabolite repression in *P. aeruginosa*. The regulatory RNA CrcZ, which is up-regulated during growth of *P. aeruginosa* on less preferred carbon sources, is known to sequester Hfq, which relieves Hfq-mediated translational repression of genes. The differential carbapenem susceptibility during growth on different carbon sources can thus be understood in light of Hfq-dependent *oprD/opdP* regulation and of the antagonizing function of the CrcZ RNA on Hfq regulatory complexes.

Keywords: *Pseudomonas aeruginosa*, *opdP*, *oprD*, carbapenem resistance, riboregulation, catabolite repression

INTRODUCTION

The opportunistic human pathogen *Pseudomonas aeruginosa* can cause severe infections. It is particularly devastating for immunocompromised individuals and patients with cystic fibrosis, leading to high morbidity and mortality. As they are less prone to degradation by extended spectrum β -lactamases, carbapenems are frequently used to treat severe infections of Gram-negative bacteria including *P. aeruginosa* (Papp-Wallace et al., 2011; Fritzenwanker et al., 2018). Carbapenem-resistant *P. aeruginosa* strains are increasingly occurring (McDougall et al., 2013; Castanheira et al., 2014; Buehrle et al., 2017), which prompted the World Health Organization to rank carbapenem-resistant *P. aeruginosa* among the priority pathogens to investigate new drug treatments (Tacconelli et al., 2018). *P. aeruginosa* exhibits several resistance mechanisms toward carbapenems including the production of metallo- β -lactamases and carbapenemase (Sacha et al., 2008; Bassetti et al., 2018) as well as dedicated efflux systems (Chalhoub et al., 2016). Another notable feature of *P. aeruginosa*'s high intrinsic antibiotic resistance is the low outer membrane permeability (Hancock and Woodruff, 1988; Breidenstein et al., 2011). *P. aeruginosa* utilizes

a variety of specialized outer membrane porins (Hancock and Brinkman, 2002; Eren et al., 2012). Two of them, OprD/OccD1 and OpdP/OccD3, show high sequence similarity (51%) and serve as entry ports for basic amino acids and small peptides as well as for certain carbapenems, e.g., imipenem and meropenem (Quinn et al., 1986; Trias et al., 1989; Tamber and Hancock, 2006; Papp-Wallace et al., 2011; Isabella et al., 2015). In fact, the first documented case of clinical resistance to carbapenems was found to be due to a loss of the monocistronic *oprD* gene (Quinn et al., 1986). The deletion of *oprD* resulted in a decreased susceptibility to carbapenems, but deletion of the *opdP* gene alone, residing in an operon together with genes encoding a dipeptide ABC transport system (Chevalier et al., 2017), did not cause a significant change. However, the deletion of both genes led to a remarkable increase in resistance when compared to the deletion of *oprD* alone (Isabella et al., 2015).

The RNA chaperone Hfq is a pleiotropic regulator and virulence factor in *P. aeruginosa* (Sonnleitner et al., 2003, 2006, 2018). Hfq is involved in the control of mRNA translation through distinct mechanisms. In riboregulation, Hfq can act indirectly by facilitating base-pairing interactions of small regulatory RNAs (sRNAs) with cognate mRNA targets (Vogel and Luisi, 2011; Kavita et al., 2018). On the other hand, Hfq can directly repress translation, by binding to A-rich sequences at or in the vicinity of translation initiation sites (Sonnleitner and Bläsi, 2014). Hfq has several distinct RNA binding sites. Crystallographic and biophysical data showed that RNA recognition is mediated by distinct interactions with distal, proximal, and rim faces of the hexameric ring (Schumacher et al., 2002; Link et al., 2009; Sauer et al., 2012; Panja et al., 2013). Many sRNAs bind to the proximal side of Hfq via U-rich stretches (Schumacher et al., 2002; Mikulecky et al., 2004; Link et al., 2009) or through the poly-uridine tails derived from rho-independent terminators (Otaka et al., 2011; Sauer and Weichenrieder, 2011; Ishikawa et al., 2012). Internal U/A-rich regions in sRNAs as well as in mRNAs were found to interact with arginine patches on the lateral rim of the Hfq-hexamer (Sauer et al., 2012; Peng et al., 2014; Schu et al., 2015). The distal side recognizes A-rich regions, previously defined as ARN repeats, where A is an adenine, R is any purine nucleotide and N is any nucleotide, which are often present in mRNAs around the ribosome binding site (Link et al., 2009; Murina et al., 2013; Robinson et al., 2014; Sonnleitner and Bläsi, 2014).

A GRIL-seq approach identified two base-pairing small regulatory sRNAs, Sr0161, and EsrA, as negative translational regulators of *oprD* (Zhang et al., 2017). EsrA is transcriptionally controlled by the envelope stress response regulator AlgU/T (Ferrara et al., 2015), and its expression is further up-regulated after a shift from high to low oxygen supply, and upon entry into stationary phase (Ferrara et al., 2015; Zhang et al., 2017). Sr0161 did not show any phase dependent expression in full broth (Zhang et al., 2017). The study by Zhang et al. (2017) also suggested an interaction between Sr0161 and *opdP* mRNA. RT-qPCR showed that the *opdP* levels were decreased after overexpression of the sRNA and modestly increased in a *sr0161* deletion mutant (Zhang et al., 2017). However, in contrast to Hfq-Sr0161/EsrA-mediated negative translational regulation of *oprD*,

it remains unknown whether Sr0161 directly regulates translation of *opdP* (Zhang et al., 2017).

In *P. aeruginosa*, carbon catabolite repression (CCR) operates at the post-transcriptional level. In the presence of preferred C-sources (e.g., succinate), the RNA chaperone Hfq binds to the translation initiation region (TIR) of mRNAs encoding proteins important for the uptake and utilization of less preferred C-sources, and represses their translation (Sonnleitner and Bläsi, 2014). Upon binding, the Hfq/RNA complex forms a “platform” to which the catabolite repression protein Crc can bind, which results in stabilization of the repressive complex (Sonnleitner et al., 2018; Pei et al., 2019). When the preferred C-source is exhausted, the levels of the Hfq-titrating RNA CrcZ increase, which in turn leads to relieve of Hfq/Crc-mediated CCR (Sonnleitner and Bläsi, 2014). Previous studies provided some hints that *oprD* is regulated by CCR (Ochs et al., 1999; Linares et al., 2010). However, recent omics studies are somewhat inconsistent. A ChIP-seq approach identified *oprD* mRNA among the mRNAs that were co-immunoprecipitated with antibodies directed against Hfq as well as against Crc (Kambara et al., 2018). Nevertheless, transcriptomic and proteomic analyses designed to reveal the Crc regulon revealed only *opdP* as a candidate for Crc-mediated post-transcriptional regulation but not *oprD* (Corona et al., 2018).

Taken these studies together with the observation that a PAO1*hfq*-deletion mutant was more susceptible to carbapenems when compared with the parental strain (Ducret et al., 2016; Pusic et al., 2018), it appeared safe to assume that Hfq is involved in regulation of both, *oprD* and *opdP*. In this study, we addressed the question whether post-transcriptional regulation of *oprD* and *opdP* occurs through riboregulation and/or through CCR, i.e., through Hfq/Crc repressive complexes. To dissect the regulatory mechanism(s) for *oprD* and *opdP*, we used a genetic approach employing translational reporter constructs in conjunction with different *P. aeruginosa* O1 mutant strains. These studies verify that *oprD* is regulated by Hfq-mediated riboregulation and strongly indicate that *opdP* is directly translationally repressed by Hfq/Crc.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Strains and plasmids used in this study are listed in **Supplementary Table S1**. If not indicated otherwise, the cultures were grown at 37°C in either Lysogeny-broth (LB) (Sambrook et al., 1989) or basal-salt medium (BSM) supplemented with the indicated carbon sources (Sonnleitner et al., 2009). When required, the following concentrations of antibiotics were used: 15 µg/ml gentamicin, 25 µg/ml tetracycline, and 100 µg/ml ampicillin for *Escherichia coli*; 50 µg/ml gentamicin, 100 µg/ml of tetracycline, and 250 µg/ml carbenicillin for *P. aeruginosa*.

Construction of PAO1 Deletion Strains

The deletion of the first 82 nucleotides (nt) of *ersA* (coordinates 6183580-6183661 of the PAO1 genome; Winsor et al., 2016)

and the deletion of the *sr0161* 3' end including additional 24 nt downstream of *sr0161* (coordinates 184302–184482 of the PAO1 genome; Winsor et al., 2016) was created by homologous recombination, respectively (Zhang et al., 2017). Briefly, plasmids pEXG2-*sr0161* (for generation of PAO1Δ*sr0161* and PAO1Δ*hfq*Δ*sr0161*) and pEXG2-*ersA* (for generation of PAO1Δ*ersA*Δ*sr0161* and PAO1Δ*hfq*Δ*ersA*Δ*sr0161*) were mobilized into the strains PAO1, PAO1Δ*hfq*, PAO1Δ*sr0161*, and PAO1Δ*hfq*Δ*sr0161* with the aid of *E. coli* strain S17-1, and then chromosomally integrated through selection for gentamicin. Excision of the vector by a second crossover event was achieved by selection of sucrose insensitive cells as the pEXG2 vector encodes the *Bacillus subtilis* *sacB* gene, whose gene product – levan sucrose – renders Gram-negative cells sensitive to sucrose (Rietsch et al., 2005; Hmelo et al., 2015).

Construction of Plasmid pME6015P_{tac}

For construction of a cloning vector suited for the generation of translational *lacZ* fusions transcribed under the control of the P_{tac} promoter, a 1,522-base pair (bp) fragment encoding the LacI^q repressor gene and the P_{tac} promoter together with the operator sites located downstream of the transcriptional start site were amplified by PCR using the oligonucleotides L85 (5'-GAT ATC GAA TTC GAA CGC CAG CAA GAC-3') and M85 (5'-TTT TTG GAT CCA ATT GTT ATC CGC TCA CAA TTC C-3') and plasmid pME6032 as template. The PCR fragment was cleaved with *Eco*RI and *Bam*HI, and then ligated into the corresponding sites of plasmid pME6015.

Construction of Plasmids pME6015P_{tac}*oprD*::*lacZ* and pME6015P_{tac}*opdP*::*lacZ*

To construct translational gene fusions between *oprD* and *opdP*, respectively, and *lacZ* under transcriptional control of the P_{tac} promoter, a 71-bp fragment of the TIR of *oprD* (coordinates 1045365–1045294 of the PAO1 genome; Winsor et al., 2016) and a 118-bp fragment of the TIR of *opdP* (coordinates 5038800–5038918 of the PAO1 genome; Winsor et al., 2016) were amplified by PCR using the oligonucleotide pairs A123 (5'-ACG TGG ATC CAC AAG AAG AAC TAG CCG TCA C-3')/H112 (5'-ACG TCT GCA GGC TCC ACT TCA TCA CTT TCA TTG-3') for *oprD* and Q144 (5'-ACG TGG ATC CTC GCC GCG CCG TCT TCG-3')/O144 (5'-ACG TCT GCA GAG CGT TCC TGG CGG AAC-3') for *opdP*, respectively, and chromosomal DNA of PAO1 as template. The PCR fragments were cleaved with *Bam*HI and *Pst*I, and then ligated into the corresponding sites of plasmid pME6015P_{tac}. Despite the presence of LacI^q, the P_{tac} promoter was leaky in the pME6015P_{tac} derivatives (not shown). Thus, IPTG was not added in the experiments described below.

Co-immunoprecipitation of mRNAs Bound to Hfq

A total of 40 ml of PAO1 culture was grown in BSM complex medium (Sonnleitner et al., 2018) and harvested at an OD₆₀₀ of 1.5. The cells were first washed in lysis buffer (20 mM Tris pH 8.0, 150 mM KCl, 1 mM MgCl₂, 1 mM DTT, and 0.05%

Triton X-100) and then snap frozen in liquid nitrogen. The cells were lysed by sonication (six times for 10 sec on ice) in 800 μl lysis buffer in the presence of 200 U RiboLock® RNase inhibitor (Fermentas). Cell debris were removed by centrifugation and anti-Hfq antibodies (Pineda) were added to 60 μl supernatant and incubated for 2 h at 4°C on a rotating wheel. Then, 5 μl Dynabeads® Protein G beads (Novex) were added, and the incubation was continued for 1 h. The beads were washed three times with lysis buffer and finally collected in 200 μl of lysis buffer without Triton X-100. Then, 100 μl phenol was added and the beads were shaken at 900 rpm for 30 min at room temperature. The RNA was purified by phenol-chloroform extraction, which was followed by ethanol precipitation. Libraries were constructed using NEBNext® Ultra™ Directional RNA Library Prep Kit from Illumina. A total of 100 bp single end sequence reads were generated using the Illumina HiSeq 2000 platform at the Vienna BioCenter Core Facility¹. Sequencing adapter removal was performed with cutadapt (Martin, 2011). Mapping of *oprD* and *opdP* RNA against the PAO1 reference genome (NCBI accession number NC_002516.2) was performed with Segemehl (Hoffmann et al., 2009) with default parameters. The mapped sequencing data were prepared for visualization using the ViennaNGS tool box, and visualized with the UCSC Genome Browser (Wolfinger et al., 2015). The raw sequencing data were deposited in the European nucleotide archive (ENA) as a study under the accession number PRJEB37368.

Microscale Thermophoresis

For *in vitro* transcription of the 100 nt long *oprD* RNA fragment [nt –61 to nt +39 with regard to the A (+1) of the AUG start codon] and the 150 nt long *opdP* RNA fragment [nt –111 to nt +39, with regard to the G (+1) of the GUG start codon], the AmpliScribe T7-Flash Transcription Kit (Epicentre Biotechnologies) was used according to the manufacturer's instructions. First, PCR fragments were generated with the primer pairs Y133 (5'-TCT AGA CGT AAT ACG ACT CAC TAT AGG CTA GCC GTC ACT GCG GCA C-3')/Z133 (5'-AAC CGC CAG TGC AAT GGC-3') (*oprD*) and B148 (5'-TCT AGA CGT AAT ACG ACT CAC TAT AGG CCA GGA GCG CTC GCC TC-3')/C148 (5'-TCC CAG CGT CGC GCC GGT C-3') (*opdP*), and *P. aeruginosa* chromosomal DNA. The forward primers contained a T7 promoter sequence (underlined).

For microscale thermophoresis (MST) (Wienken et al., 2010), 20 μM of Hfq protein was labeled with Monolith NT™ Protein Labelling Kit RED-NHS according to the manufacturer's instructions (Nanotemper Technologies). For determination of protein-RNA interactions, 56 nM labeled Hfq was used in the presence of increasing concentrations of non-labeled *in vitro* transcribed RNA (either *oprD*_{–61 +39} or *opdP*_{–111 +39}). The ligands were dissolved in MST-buffer (50 mM Tris pH 7.4, 150 mM NaCl, and 10 mM MgCl₂) in the presence of 0.1% Tween-20 and 0.1% BSA. After 30 min incubation at 37°C, the samples were loaded onto NT.115 hydrophilic capillaries (Nanotemper Technologies) and measured in an MST Monolith

¹<https://www.viennabiocenter.org/facilities/>

NT.115 Green/Red instrument at the Vienna BioCenter Core Facility². The MST measurements were performed in duplicate. The data for MST analysis were recorded at 25°C using the red LED (excitation: 625 nm and emission: 680 nm); MST Power 40%, LED Power 50%. Data analysis was performed with NTAffinityAnalysis v2.0.2 for thermophoresis and T-jump analysis 0 and 5 s after the pulse. For determination of the K_d -values the Hill-fit model within the NTAffinityAnalysis software was used.

Agar Disk Diffusion Assay

PAO1 and mutants thereof were grown in BSM supplemented with either 40 mM succinate or mannitol to an OD₆₀₀ of 1.8–2.0. Then, 200 µl of cultures were plated on agar plates containing the respective media and filter disks loaded with 10 µg imipenem were applied on top (Oxoid). The plates were incubated at 37°C. The diameter of the growth inhibition zones was measured in mm.

β-Galactosidase Assays

The β-galactosidase activities were determined as described (Miller, 1972). The cells were permeabilized with 5% toluene. Unless indicated otherwise, the β-galactosidase units in the different experiments were derived from at least two independent experiments, and are shown as mean. The error bars in the different figures represent standard deviations. Except for the data in **Figure 3**, statistical analyses were performed in Excel with a two tailed distributed Student's *T*-test of two sample arrays with unequal variance, ns (non-significant); $P > 0.05$, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. Due to the multiple comparison in **Figure 3**, the results were statistically analyzed by an ANOVA test with *post hoc* multiple comparison. In short, Levene's test was used to test for equality (homogeneity) of variances between the tested groups. Statistical significance was determined by one-way ANOVA with the Tukey's *post hoc* test when more than two groups with normal distribution were compared (**Figure 3A**). When more than two groups with unequal variance were compared, the Brown-Forsythe and Welch's ANOVA test with the Dunnett's T3 *post hoc* test was used (**Figures 3B–D**), ns (non-significant); $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant results.

Western-Blot Analyses

Equal amounts of total proteins were separated on a 12% SDS-polyacrylamide gel, and then electro-blotted onto a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, and probed with rabbit anti-Hfq, rabbit anti-Crc or rabbit anti-*E. coli*-S1 antibodies (Sonnleitner and Bläsi, 2014). Immunodetection of ribosomal protein S1 served as a loading control. The antibody–antigen complexes were visualized with horseradish peroxidase (HRP) conjugated anti-rabbit antibodies (Cell Signaling Technology) using the SuperSignalTM West Pico PLUS chemiluminescent substrate kit (Thermo Scientific). The signals were detected with ChemiDocTM Touch Imaging System (BioRad) and analyzed with ImageLab 5.2.1 (BioRad).

²<https://www.viennabiocenter.org/facilities/>

RESULTS

Hfq Is Involved in Translational Regulation of *oprD* and *opdP*

Recent studies revealed that *P. aeruginosa* *hfq* deletion mutants showed an increased susceptibility to imipenem (Ducret et al., 2016; Pusic et al., 2018), which coincided with higher *oprD* and *opdP* transcript levels in the *hfq* mutant strain when compared with the parental strain during growth in different media (**Supplementary Figure S1** and **Supplementary Table S2**) (Ducret et al., 2016; Pusic et al., 2018). To verify the impact of Hfq on *oprD* and *opdP* translation, *oprD::lacZ* and *opdP::lacZ* translational gene fusions were constructed and the β-galactosidase activities conferred by the respective fusion proteins were determined in strains PAO1 and PAO1Δ*hfq* harboring plasmids pME6015P_{tac}*oprD::lacZ* and pME6015P_{tac}*opdP::lacZ*, respectively. Transcription of either chimeric gene was driven by the P_{tac} promoter to control for potential transcriptional effects of Hfq (Ducret et al., 2016). The strains were grown in LB medium to an OD₆₀₀ of 2.0, as the sRNAs ErsA and Sr0161 are transcribed under these conditions (Zhang et al., 2017). As reported by Zhang et al. (2017), our RNAseq analysis confirmed growth independent quantities of Sr0161 RNA in LB medium and increased levels of ErsA RNA in stationary phase when compared to logarithmically growing cells (**Supplementary Figure S2**). Translation of both reporter fusions was repressed in the presence of Hfq. However, the effect of Hfq on *oprD::lacZ* translation was less pronounced (**Figure 1A**; 1.5-fold difference between PAO1 and PAO1Δ*hfq* harboring pME1615P_{tac}*oprD::lacZ*) than on *opdP::lacZ* translation (**Figure 1B**; 10-fold difference between PAO1 and PAO1Δ*hfq* harboring pME1615P_{tac}*opdP::lacZ*). The differential translation rates observed for *oprD::lacZ* and *opdP::lacZ* in the wild-type strain and the corresponding *hfq* deletion strains coincided with a ~2–3-fold and ≥10-fold higher transcript abundance of *oprD* and *opdP*, respectively, in PAO1 and PAO1Δ*hfq* during cultivation in different media (**Supplementary Table S2**). As anticipated, the complementation of *hfq* by ectopic expression of a plasmid borne *hfq*_{Flag} allele in strain PAO1Δ*hfq* resulted again in repression of the *oprD::lacZ* and *opdP::lacZ* reporter genes (**Figures 1C,D**), respectively.

As the mRNA sequences of the TIRs of the *oprD* and *opdP* genes in the clinical isolate PA14 showed only minor differences to PAO1 (Winsor et al., 2016), the same experiments were performed with the clinical isolate PA14 and the corresponding PA14Δ*hfq* mutant harboring plasmids pME1615P_{tac}*oprD::lacZ* and pME1615P_{tac}*opdP::lacZ*, respectively, after growth in synthetic cystic fibrosis medium (SCFM) containing 100 µM FeSO₄ (Palmer et al., 2007; Tata et al., 2016). The outcome of these experiments was comparable with the results obtained for PAO1 after growth in LB medium (**Supplementary Figures S3A–D**).

Binding of Hfq to the Translation Initiation Regions of *oprD* and *opdP*

To test whether Hfq binds to the TIRs of *oprD* and *opdP*, a co-immunoprecipitation assay with Hfq specific antibodies was

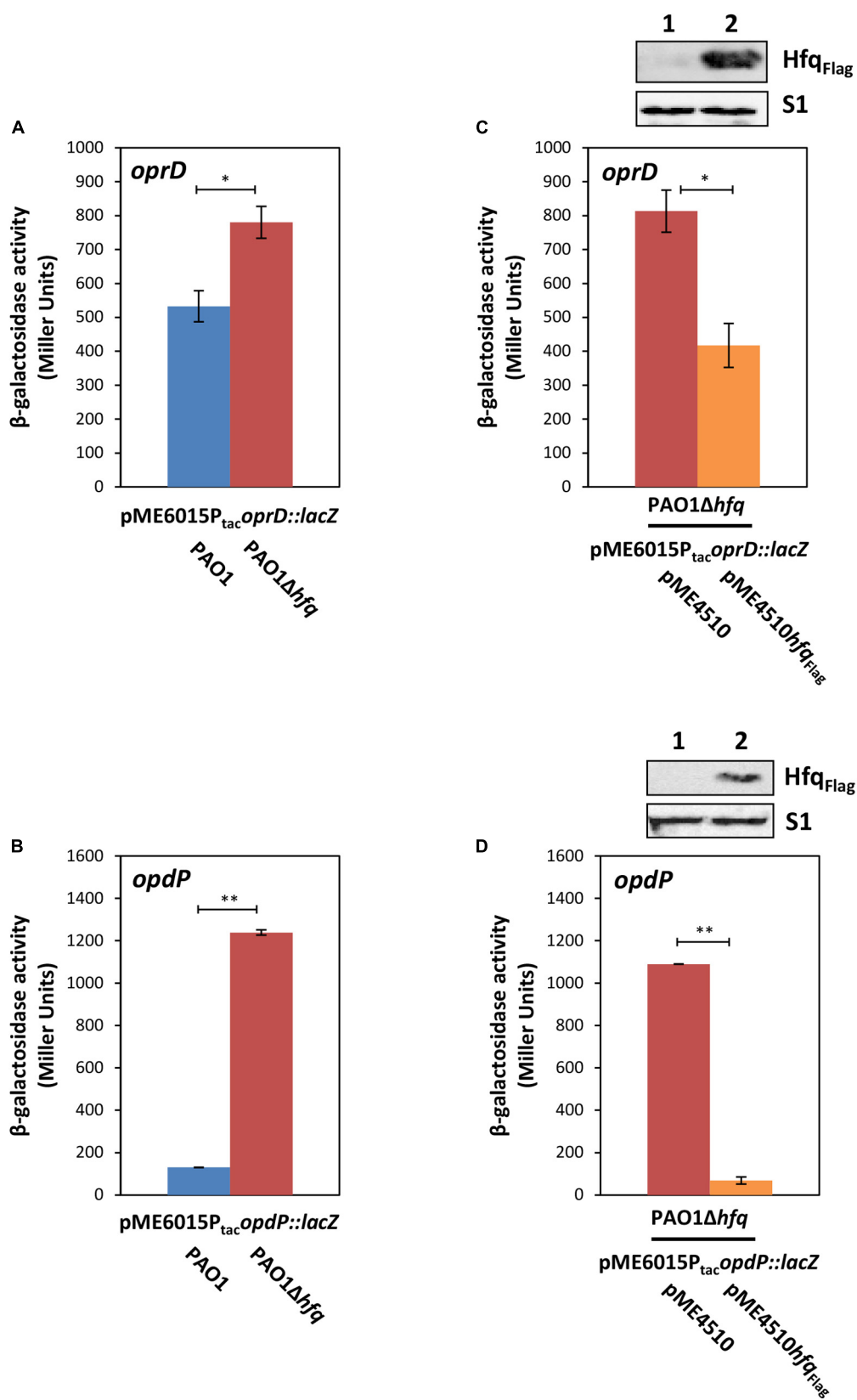


FIGURE 1 | Continued

FIGURE 1 | Hfq negatively regulates *oprD* and *opdP* translation. **(A,B)** The strains PAO1 (blue bar) and PAO1 Δ hfq (red bar) harboring either plasmid pME6015P_{tac}*oprD::lacZ* **(A)** or pME6015P_{tac}*opdP::lacZ* **(B)** were grown in LB medium. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β -galactosidase values conferred by the *oprD::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*oprD::lacZ* **(A)** and by the *opdP::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*opdP::lacZ* **(B)**, respectively. The error bars represent standard deviations from two independent experiments. The strains PAO1 Δ hfq(pME4510) (red bar) and PAO1 Δ hfq(pME4510hfq_{Flag}) (orange bar) harboring either plasmid pME6015P_{tac}*oprD::lacZ* **(C)** or pME6015P_{tac}*opdP::lacZ* **(D)** were grown in LB medium. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β -galactosidase values conferred by the *oprD::lacZ* translational fusion gene **(C)** and by the *opdP::lacZ* translational fusion gene **(D)**, respectively, in the presence or absence of ectopic hfq_{Flag} expression. The error bars represent standard deviations from two independent experiments. Statistical analyses were performed in Excel with a two tailed distributed Student's *T*-test of two sample arrays with unequal variance. ns $P > 0.05$, * $P \leq 0.05$, and ** $P \leq 0.01$. **(C,D: top panels)**, Hfq and S1 levels in strains PAO1 Δ hfq(pME4510) (lane 1) and PAO1 Δ hfq(pME4510hfq_{Flag}) (lane 2) harboring either plasmid pME6015P_{tac}*oprD::lacZ* **(C)** or pME6015P_{tac}*opdP::lacZ* **(D)**. The Hfq levels were determined by Western-blot analyses using anti-Hfq antibodies. Immunodetection of ribosomal protein S1 served as a loading control.

performed to identify protein bound RNA fragments. Subsequent RNA-seq revealed a distinct sub-sequence upstream of the *oprD* start codon (**Figure 2A**). This region contains several ARN motifs, which have been shown to bind to the distal side of Hfq (Link et al., 2009). The sub-sequence also comprises the ErsA and Sr0161 interaction sites (**Figure 2A**) (Zhang et al., 2017).

The co-immunoprecipitated RNA sub-sequence including the start codon of *opdP* comprised as well several ARN-triplets and overlaps with the predicted Sr0161 binding-site (**Figure 2B**) (Zhang et al., 2017).

The binding affinity of Hfq for the respective co-immunoprecipitated *oprD* and *opdP* sub-sequences was determined by MST. Hfq exerted a high affinity for the *oprD*_{-61 -+ 39} sub-sequence ($K_d = 19.7 \pm 2.8$ nM) and a somewhat lower affinity for the *opdP*_{-111 -+ 39} sub-sequence ($K_d = 78.1 \pm 1.97$ nM) (**Figure 2C**).

At this junction it seems worth noting that binding of Hfq to the TIRs of either *oprD* or *opdP* is anticipated regardless of whether Hfq/sRNA-mediated regulation is the underlying mechanism of translational repression, or whether this occurs through Hfq/Crc repressive complexes.

The sRNA Sr0161 Does Not Impact Translation of *opdP*

Next, we re-examined whether sRNA Sr0161, which was identified by GRIL-seq and suggested to base-pair with the *opdP* TIR (Zhang et al., 2017) can translationally regulate *opdP*. The strains PAO1 and PAO1 Δ Sr0161 harboring plasmid pME6016P_{tac}*opdP::lacZ* were grown in LB medium to an OD₆₀₀ of 2.0. The β -galactosidase activities conferred by the translational *opdP::lacZ* reporter gene were indistinguishable in either strain (**Figure 3A**). In addition, ectopic expression of Sr0161 in PAO1 and PAO1 Δ Sr0161 harboring plasmid pME6016P_{tac}*opdP::lacZ* did not result in significant translational repression of the *opdP::lacZ* reporter gene (**Figure 3B**), arguing against translational regulation of *opdP* by Sr0161. However, the β -galactosidase activity conferred by the *opdP::lacZ* reporter gene encoded by plasmid pME6015P_{tac}*opdP::lacZ* was approximately 10-times higher in strain PAO1 Δ hfq Δ Sr0161 when compared with strains PAO1 and PAO1 Δ Sr0161 (**Figure 3A**), suggesting that Hfq regulates *opdP* either with the aid of an hitherto unknown sRNA or directly by a Hfq/Crc repressive complex.

On the other hand, our studies with the constructed PAO1 Δ ErsA Δ Sr0161(pME6015P_{tac}*oprD::lacZ*) double mutant and with the PAO1 hfq Δ ErsA Δ Sr0161(pME6015P_{tac}*oprD::lacZ*)

triple mutant strain showed that the absence of the sRNAs leads to de-repression of *oprD::lacZ* translation in both strains (**Figure 3C**), whereas ectopic overexpression of either sRNA restored translational repression of *oprD::lacZ* in strain PAO1 Δ ErsA Δ Sr0161(pME6015P_{tac}*oprD::lacZ*) to the same level but not in strain PAO1 Δ hfq Δ ErsA Δ Sr0161(pME6015P_{tac}*oprD::lacZ*) (**Figure 3D**). Thus, the latter studies corroborate and extend the observations of Zhang et al. (2017) in that (i) EsrA and Sr0161 negatively regulate *oprD* translation and that (ii) not only Sr0161 operates in a Hfq-dependent manner but also EsrA.

Crc Impacts Hfq-Mediated *opdP* Regulation but Not on Hfq/EsrA/Sr0161-Mediated Riboregulation of *oprD*

Previous studies have shown that both, Hfq and Crc, are required for tight translational repression of mRNAs, which are subject to CCR (Sonnleitner and Bläsi, 2014; Moreno et al., 2015). Although the presence of Crc did not significantly enhance the affinity of Hfq for a RNA substrate, the simultaneous interactions of Crc with both binding partners resulted in an Hfq/Crc/RNA assembly with increased stability when compared with the Hfq/RNA complex alone (Sonnleitner et al., 2018). In other words, Crc can be regarded a co-repressor in Hfq/Crc repressive complexes. A recent structural study provided a rationale for the increased stability of the Hfq/Crc/RNA assembly by showing that the Hfq binding site on mRNA is sandwiched between both binding partners (Pei et al., 2019), which in turn can be readily reconciled with an increased translational repression observed for Hfq/Crc regulated genes in the presence of Crc (wild type strain) when compared to an isogenic *crc* deletion strain (Sonnleitner and Bläsi, 2014). Therefore, the following experiments were performed with the rationale that Crc should impact translation of genes that are assumed to be directly controlled by a Hfq/Crc repressive complex such as *opdP* rather than genes subject to canonical riboregulation such as *oprD*. Translation of *opdP* and *oprD* was monitored in strains PAO1 and PAO1 Δ crc transformed with either plasmid pME6015P_{tac}*opdP::lacZ* or pME6015P_{tac}*oprD::lacZ*. As shown in **Figure 4A**, the β -galactosidase activity conferred by the *opdP::lacZ* translation was increased in the absence of Crc, i.e., the presence of Crc resulted in a stronger repression of *opdP::lacZ*. In contrast, the β -galactosidase activity conferred by

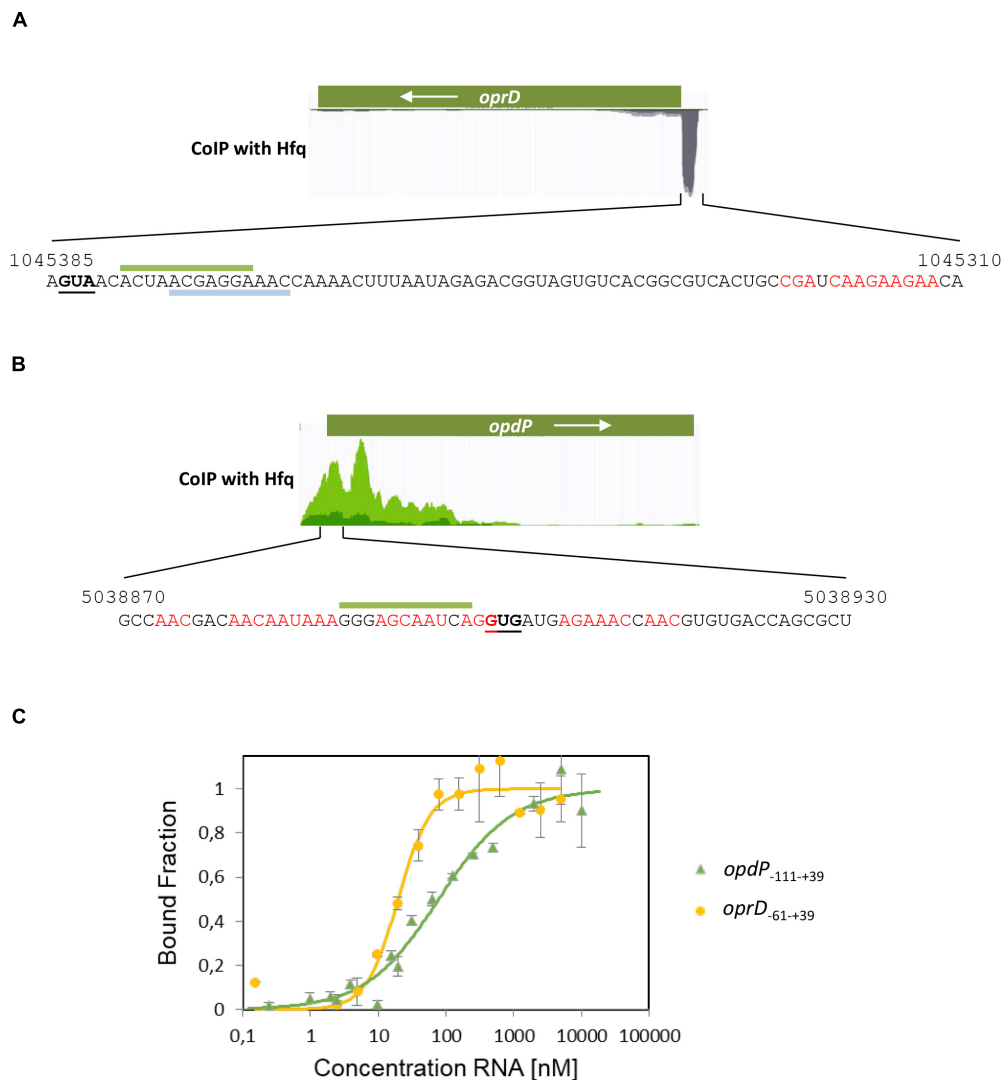


FIGURE 2 | Co-immunoprecipitation of *oprD* and *opdP* mRNA fragments with Hfq. Hfq-bound RNAs were isolated from lysates of the PAO1 culture after co-immunoprecipitation with Hfq-specific antibodies as described in section “Materials and Methods.” The identity of Hfq-bound RNAs in the supernatant was revealed by RNA-seq. **(A,B)** The read coverage visualized in the Genome Browser is shown for *oprD* **(A)** and *opdP* **(B)**, respectively. The sequences of the co-immunoprecipitated mRNA fragments corresponding to the TIR of *oprD* **(A)** and *opdP* **(B)**, respectively, are shown below. The numbers refer to the PAO1 genome coordinates (<http://www.pseudomonas.com>) (Winsor et al., 2016). The start codons of *oprD* and *opdP* are underlined. Putative Hfq binding sites are indicated in red. The interacting sequences for EsrA and Sr0161 in the *oprD* TIR **(A)** (Zhang et al., 2017) are indicated by gray and green bars, respectively. The putative interacting sequence for Sr0161 in the *opdP* TIR **(B)** is indicated by a green bar **(B)** (Zhang et al., 2017). **(C)** Microscale thermophoresis reveals the K_d of Hfq for *oprD*_{-61 to +39} RNA (yellow circles) and *opdP*_{-111 to +39} RNA (green triangles) with 19.7 ± 2.8 nM and 78.1 ± 1.97 nM, respectively. Increasing amounts of the non-labeled *in vitro* transcribed *oprD*_{-61 to +39} and *opdP*_{-111 to +39} fragments were added to 56 nM fluorescently labeled Hfq protein. The dissociation constant (K_d) of *oprD*_{-61 to +39} and *opdP*_{-111 to +39} was determined as described in section “Materials and Methods,” and was expressed as mean EC50 $\pm$ EC50 confidence interval of two independent experiments. Thermophoresis/T-jump analysis is shown. LED power of 50% and MST power of 40% were used.

the *oprD*::*lacZ* reporter gene was unaffected by Crc (**Figure 4B**). In line, ectopic expression of *crc-flag* resulted in repression of *opdP*::*lacZ* in strain PAO1 Δ *crc*(pME6015P_{tac}*opdP*::*lacZ*) (**Figure 4C**), whereas ectopic expression of *crc-flag* did not significantly change *oprD*::*lacZ* translation in strain PAO1 Δ *crc*(pME6015P_{tac}*oprD*::*lacZ*) (**Figure 4D**). These data strongly suggested that Crc contributes to Hfq-mediated direct translational regulation of *opdP*, i.e., that its translation is controlled by a Hfq/Crc repressive complex. As translational

regulation of *oprD* was rather independent of Crc, these studies further support the notion that translational regulation of *oprD* is only subject to Hfq/EsrA/Sr0161-mediated riboregulation.

High CrcZ Levels Increase *oprD* and *opdP* Translation

Transcription of the Hfq titrating RNA CrcZ is known to be induced by less preferred carbon sources such as mannitol

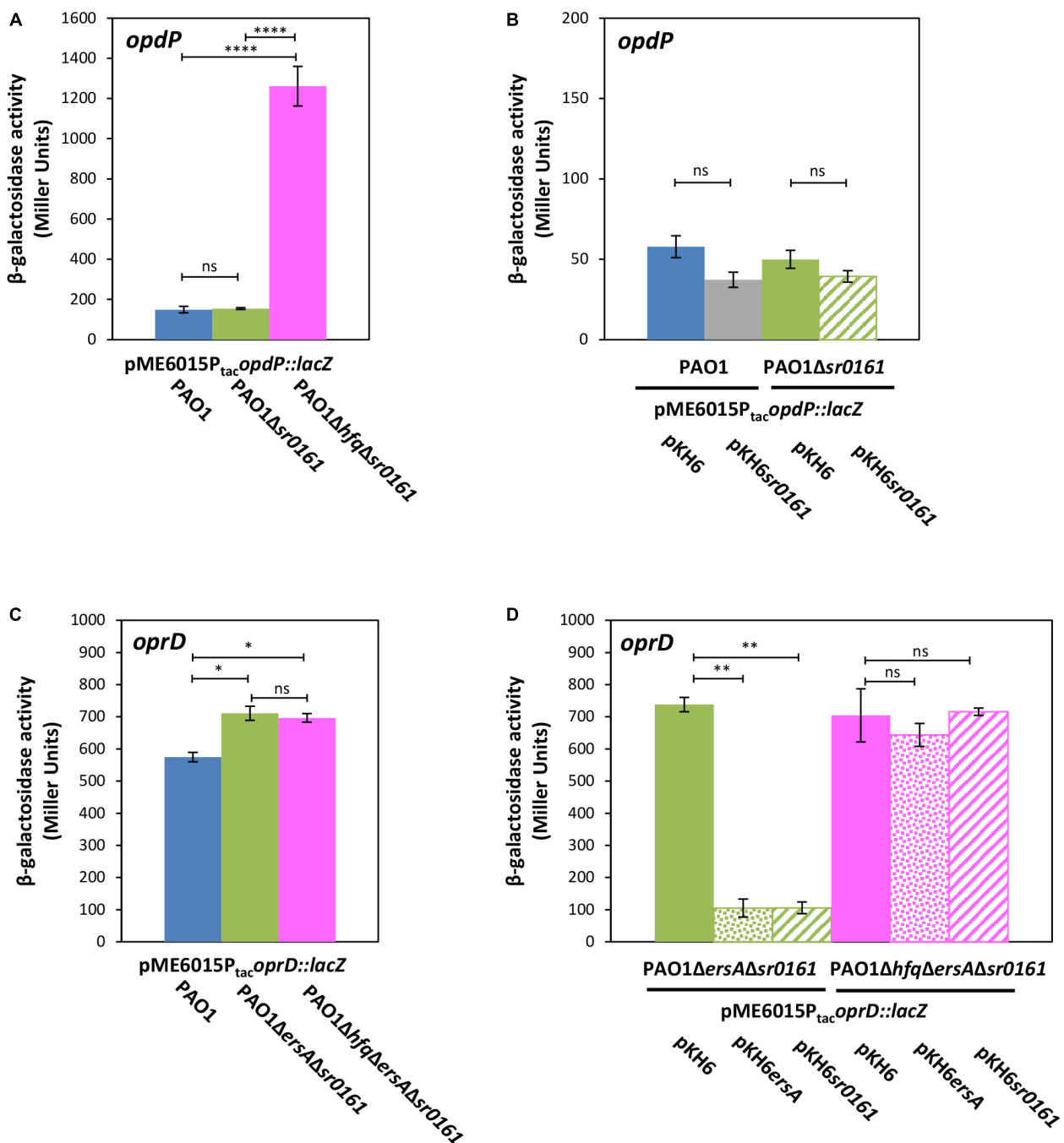


FIGURE 3 | The sRNAs *Sr0161* does not regulate *opdP* translation. **(A)** The strains PAO1 (blue bar), PAO1Δ*sr0161* (green bar), and PAO1Δ*hfq*Δ*sr0161* (pink bar) harboring plasmid pME6015P_{tac} *opdP::lacZ* were grown in LB medium. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β-galactosidase values conferred by the *opdP::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac} *opdP::lacZ*. **(B)** The strains PAO1 (pKH6) (blue bar), PAO1 (pKH6*sr0161*) (gray bar), PAO1Δ*sr0161* (pKH6) (green bar), and PAO1Δ*sr0161* (pKH6*sr0161*) (striped green bar) harboring plasmid pME6015P_{tac} *opdP::lacZ* were grown in LB medium supplemented with 0.2% arabinose to induce sRNA gene expression from plasmid pKH6*sr0161*. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β-galactosidase values conferred by the *opdP::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac} *opdP::lacZ*. **(C)** The strains PAO1 (blue bar), PAO1Δ*ersA*Δ*sr0161* (green bar) and PAO1Δ*hfq*Δ*ersA*Δ*sr0161* (pink bar) harboring plasmid pME6015P_{tac} *oprD::lacZ* were grown in LB medium. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β-galactosidase values conferred by the *oprD::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac} *oprD::lacZ*. **(D)** Ectopic expression of *ersA* and *sr0161* in strains PAO1Δ*ersA*Δ*sr0161* (green bars) and PAO1Δ*hfq*Δ*ersA*Δ*sr0161* (pink bars) harboring plasmid pME6015P_{tac} *oprD::lacZ*. The β-galactosidase values conferred by the *oprD::lacZ* translational fusion gene in either strain in the absence of *ersA*/*sr0161* expression (solid bars) and in the presence of *ersA* (dotted bars) or *sr0161* (striped bars) are indicated by bars. Statistical analyses were performed with AGOVA test with *post hoc* multiple comparison as described in section “Materials and Methods.” ns (non-significant); **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

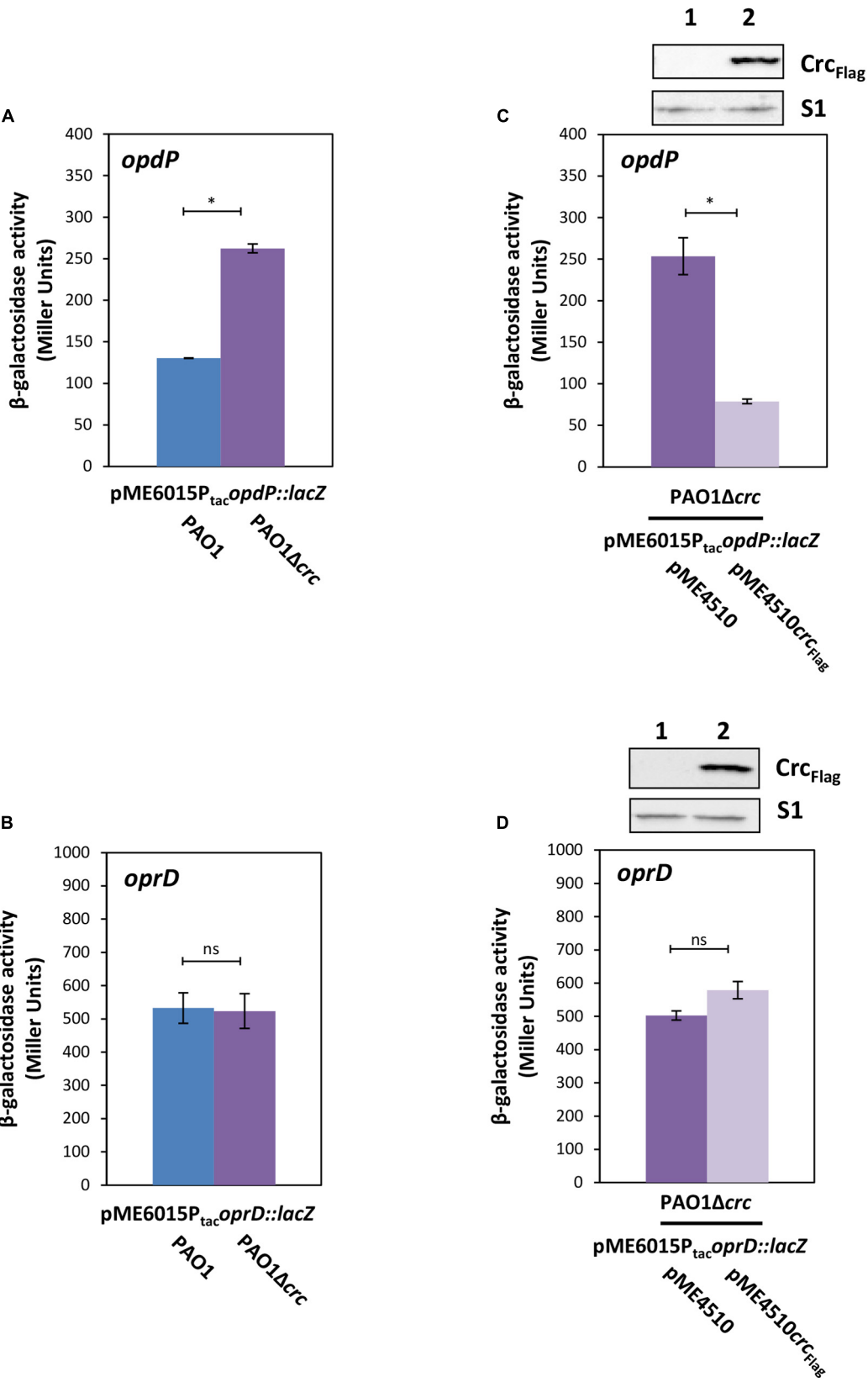


FIGURE 4 | Continued

FIGURE 4 | *Crc* impacts on *opdP* but not on *oprD* translation. **(A,B)** The strains PAO1 (blue bar) and PAO1Δ*crc* (purple bar) harboring either plasmid pME6015P_{tac}*opdP*::*lacZ* **(A)** or pME6015P_{tac}*oprD*::*lacZ* **(B)** were grown in LB medium. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β-galactosidase values conferred by the *opdP*::*lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*opdP*::*lacZ* **(A)** and by the *oprD*::*lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*oprD*::*lacZ* **(B)**. The error bars represent standard deviations from two and three independent experiments, respectively. **(C,D)** The strains PAO1Δ*crc*(pME4510) (purple bar) and PAO1Δ*crc*(pME4510*crc*_{Flag}) (light purple bar) harboring either plasmid pME6015P_{tac}*opdP*::*lacZ* **(C)** or pME6015P_{tac}*oprD*::*lacZ* **(D)** were grown in LB medium. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β-galactosidase values conferred by the *opdP*::*lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*opdP*::*lacZ* **(C)** and by the *oprD*::*lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*oprD*::*lacZ* **(D)**, respectively, in the presence or absence of ectopic *crc*_{Flag} expression. The error bars represent standard deviations from two independent experiments. Statistical analyses were performed in Excel with a two tailed distributed Student's *T*-test of two sample arrays with unequal variance. ns *P* > 0.05, **P* ≤ 0.05. Top panels, *Crc*_{Flag} and S1 levels in strains PAO1Δ*crc*(pME4510) (lane 1) and PAO1Δ*crc*(pME4510*crc*_{Flag}) (lane 2) harboring either plasmid pME6015P_{tac}*opdP*::*lacZ* **(C)** or pME6015P_{tac}*oprD*::*lacZ* **(D)**. The *Crc* levels were determined by Western-blot analyses using anti-*Crc* antibodies. Immunodetection of ribosomal protein S1 served as a loading control.

(Sonnleitner et al., 2009). Therefore, we next tested whether the susceptibility toward imipenem is increased in an *oprD* deletion strain during growth in the presence of mannitol (high levels of *CrcZ*; Sonnleitner et al., 2009) when compared to the presence of succinate (low levels of *CrcZ*; Sonnleitner et al., 2009), i.e., whether translational repression of *opdP* by Hfq/*Crc* is relieved in the presence of *CrcZ* and in the absence of *CCR*. The disk diffusion assay revealed extended growth inhibition zones for the *oprD* mutant strain during growth in mannitol (Table 1), and therefore increased susceptibility to the tested antibiotic, consistent with the notion that *opdP* translation is controlled by Hfq/*Crc*. The same experiment conducted with an *opdP* deletion strain revealed a comparable result, indicating that *CrcZ* also interferes with Hfq/*EsrA*/*Sr0161*-mediated riboregulation of *oprD*. In support, ectopic overexpression of *crcZ* from plasmid pMMB*crcZ* in strains PAO1(pME6015P_{tac}*oprD*::*lacZ*) (Supplementary Figure S4A) and PAO1(pME6015P_{tac}*opdP*::*lacZ*) (Supplementary Figure S4B) confirmed that high levels of *CrcZ* increase both *oprD* and *opdP* translation. Again, these findings are in line with a reduced minimal inhibitory concentration (MIC) of imipenem upon ectopic overexpression of *crcZ* in PAO1 (Supplementary Figure S4C).

DISCUSSION

The outer membrane porins *OprD* and *OpdP* are required for the uptake of carbapenems (Tamber and Hancock, 2006). Here, we have verified and shown that Hfq is involved in negative translational riboregulation of *oprD* by the sRNAs *EsrA* and *Sr0161*. Moreover, we have provided evidence for Hfq/*Crc*-mediated regulation of *opdP*. Hence, the increased susceptibility of *P. aeruginosa* *hfq* deletion mutants toward imipenem (Pusic

et al., 2018) can be rationalized at the molecular level by these studies. However, we cannot exclude that hitherto unknown Hfq-mediated regulatory circuits additionally impact *oprD* and *opdP* regulation.

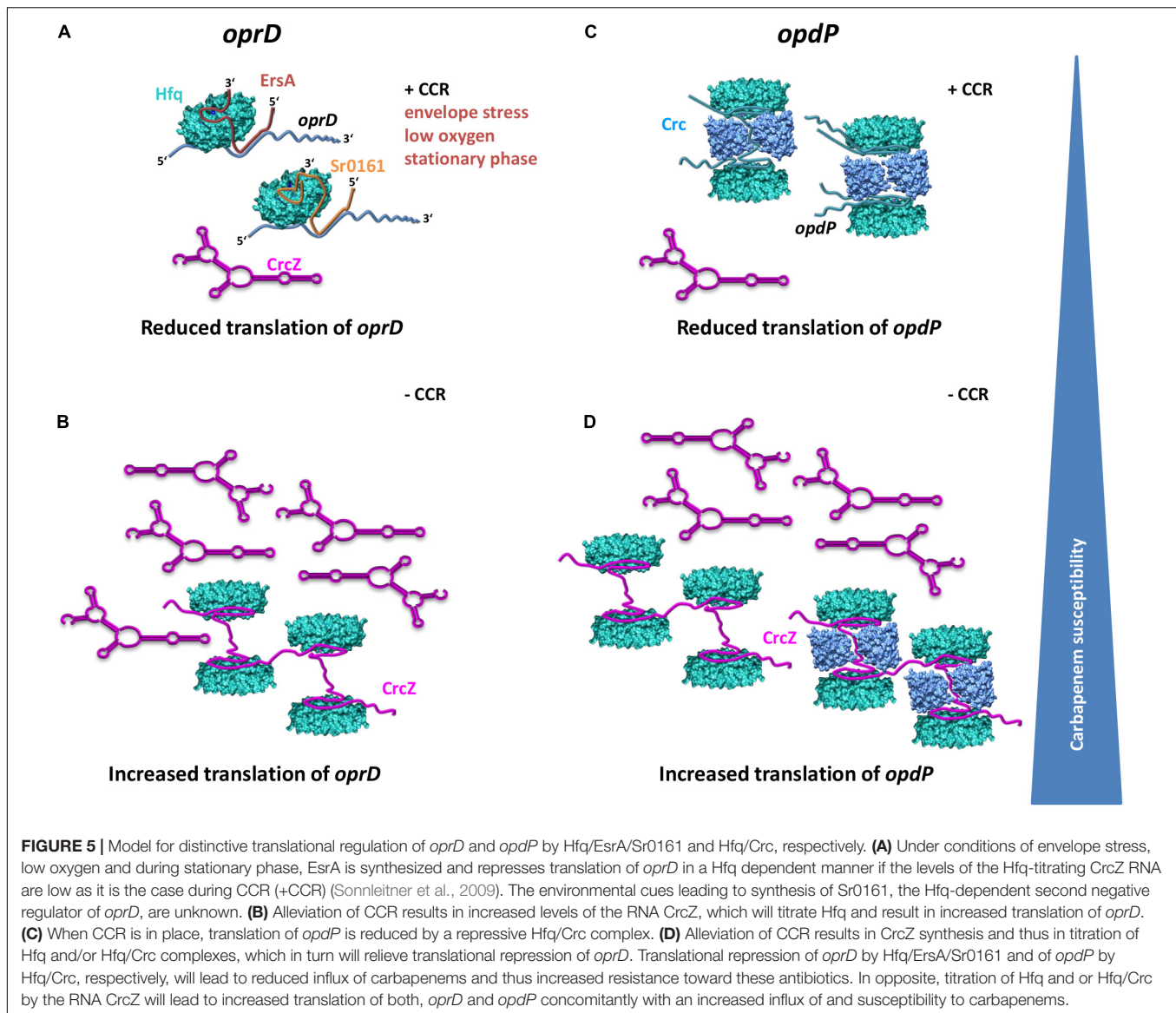
Zhang et al. (2017) observed no specific amplicons of chimeras formed by *Sr0161* and *oprD* mRNA in a Δ*hfq* strain. In addition, they showed that the *oprD* mRNA levels are increased in a Δ*hfq* strain. These experiments indicated that *Sr0161*-dependent negative regulation of *oprD* is Hfq dependent (Zhang et al., 2017). Using the triple mutant strain PAO1Δ*hfq*Δ*ersA*Δ*sr0161*(pME6015P_{tac}*oprD*::*lacZ*), we have shown directly that translational repression of *oprD*::*lacZ* upon ectopic expression of either *esrA* or *sr0161* depends on Hfq (Figure 3D). Ectopic expression of *Crc* in strain PAO1Δ*crc*(pME6015P_{tac}*oprD*::*lacZ*) insignificantly increased translation of *oprD*::*lacZ* (Figure 4D) rather than decreased it. This observation is consistent with our previous results in that over-expression of *crc* can interfere with Hfq-mediated riboregulation (Sonnleitner et al., 2018), and would argue against negative regulation of *oprD* translation by a repressive Hfq/*Crc* complex. In this context, it is also worth noting that the absence of Hfq in strain PAO1Δ*hfq*Δ*ersA*Δ*sr0161*(pME6015P_{tac}*oprD*::*lacZ*) (Figure 3C) did not result in increased de-repression of *oprD*::*lacZ* translation when compared with strain PAO1Δ*ersA*Δ*sr0161*(pME6015P_{tac}*oprD*::*lacZ*) (Figure 3C), again indicating that direct translational repression of *oprD* by Hfq/*Crc* does not occur in addition to Hfq/*EsrA*/*Sr0161*-mediated riboregulation (Figure 5A). This hypothesis also agrees with other studies (Corona et al., 2018), which indicated that *oprD* is not post-transcriptionally regulated by *Crc*.

Zhang et al. (2017) reported that deletion and over-expression of *sr0161* resulted in increased and decreased *opdP* mRNA levels, respectively, which was indicative for *Sr0161*-dependent regulation of *opdP*. However, our experiments

TABLE 1 | Sensitivity toward imipenem during growth on different carbon-sources.

Strains		PAO1	PAO1	PAO1 Δ <i>oprD</i>	PAO1 Δ <i>oprD</i>	PAO1 Δ <i>opdP</i>	PAO1 Δ <i>opdP</i>
Antibiotic	Media	BSM + 40 mM succinate	BSM + 40 mM mannitol	BSM + 40 mM succinate	BSM + 40 mM mannitol	BSM + 40 mM succinate	BSM + 40 mM mannitol
	Concentration	Inhibition zone [mm] ^a					
Imipenem	10 μg	22.25 ± 1.26	25.5 ± 1.29	18.5 ± 0.58	25.5 ± 0.58	20.5 ± 0.58	27.75 ± 2.06

^aAverage of two independent experiments ± standard deviation.



performed with the translational *opdP::lacZ* reporter gene did not reveal direct evidence for an involvement of the sRNA Sr0161 in *opdP* translation (Figures 3A,B). Hence, we can only speculate that the observations made by Zhang et al. (2017) result from indirect effects. It is also worth noting that the putative Hfq and Sr0161 binding sites appear to overlap (Figure 2B). Thus, Hfq would be assumed to compete with sRNA binding rather than support the interaction with *opdP*. Our results are rather consistent with a model wherein a repressive Hfq/Crc complex prevents *opdP* translation when CCR is in place (Figure 5C). In support, our studies showed that *opdP::lacZ* translation is de-repressed and repressed in the absence of Crc and upon ectopic expression of *crc-flag*, respectively.

Hfq is titrated by the regulatory RNA CrcZ, which abrogates its function in Hfq-Crc-mediated translational repression during CCR (Sonnleitner and Bläsi, 2014) as well as in

Hfq/sRNA-mediated riboregulation (Sonnleitner and Bläsi, 2014; Sonnleitner et al., 2017). Previous studies have shown that the levels of CrcZ are comparatively low when the cells are cultured in the presence of succinate (CCR) when compared with cells cultured in the presence of mannitol (no CCR) (Sonnleitner et al., 2009; Valentini et al., 2014). The model shown in Figure 5 therefore specifies that in the presence of low levels of CrcZ (succinate; CCR), the translation of both, *oprD* and *opdP*, is negatively regulated by Hfq/EsrA/Sr0161-mediated riboregulation (Figure 5A) and by Hfq/Crc repressive complexes (Figure 5C), respectively. In opposite, increasing levels of CrcZ (mannitol; no CCR) are anticipated to result in titration of Hfq, and in translation of *oprD* and *opdP* (Figures 5B,D). Hence, the differential imipenem susceptibility (Table 1) during growth on succinate and mannitol can thus be rationalized in light of Hfq-dependent *oprD/opdP* regulation and of the antagonizing function of the RNA CrcZ on Hfq regulatory complexes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

UB, ES, and PP conceived and designed the experiments. ES and PP performed the experiments. ES, PP, UB, and MW analyzed the data. ES and UB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.01001/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5.4.1. Supplementary Figures and Tables

Supplementary Material

Distinctive regulation of carbapenem susceptibility in *Pseudomonas aeruginosa* by Hfq

Elisabeth Sonnleitner*, Petra Pusic, Michael T. Wolfinger, and Udo Bläsi*

*** Correspondence:**

Elisabeth Sonnleitner: elisabeth.sonnleitner@univie.ac.at

Udo Bläsi: udo.blaesi@univie.ac

1 Supplementary Text

RNA-seq

Total RNA was prepared from two biological replicates of strains PAO1 and PAO1*hfq*⁻, respectively, after growth in LB medium to an OD₆₀₀ of 1.0 and 3.0. 10 ml samples were withdrawn and total RNA was extracted using the hot phenol method (Leoni et al., 1996), contaminating DNA was removed by DNase (Roche) treatment followed by phenol-chloroform (pH 5.5) extraction and ethanol precipitation. To remove ribosomal RNAs, the Ribo-Zero™ Magnetic Kit for Gram-negative bacteria (Epicentre) was used according to the manufacturer's instructions. Libraries were constructed using NEBNext® Ultra™ Directional RNA Library Prep Kit from Illumina. 100 base pair single end sequence reads were generated using the Illumina HiSeq 2000 platform at the Vienna BioCenter Core Facility (<https://www.viennabiocenter.org/facilities/>). Sequencing adapter removal was performed with cutadapt (Martin, 2011). Mapping of the samples against the PAO1 reference genome (NCBI accession number NC_002516.2) was performed with Segemehl (Hofmann et al., 2009) with default parameters. Reads mapping to regions annotated as either rRNA or tRNA were discarded from all data and ignored for all follow up analysis steps. The mapped sequencing data were prepared for visualization using the ViennaNGS tool box, and visualized with the UCSC Genome Browser (Wolfinger et al., 2015). Reads per gene were counted using BEDTools (Quinlan and Hall, 2010) and the Refseq annotation of *P. aeruginosa* (NC_002516.2). Differential gene expression analysis was performed with DESeq (Anders and Huber, 2010). The raw sequencing data were deposited in the European nucleotide archive (ENA) as a study under the accession number PRJEB37368. Normalized total reads of *oprD* and *opdP*, their adjusted P-values and their fold change of the differential gene expression analysis are presented in **Supplementary Table S2**.

Northern blot analyses

Total RNA was purified using hot phenol (Leoni et al., 1996). The steady state levels of CrcZ and 5S rRNA (loading control) were determined by Northern-blotting using 4 µg of total RNA. The RNA samples were denatured for 5 min at 65°C in loading buffer containing 50% formamide, separated on a 8% polyacrylamide/8 M urea gel, and then transferred to a nylon membrane by electro-blotting. The RNA was cross-linked to the membrane by exposure to UV light. The membranes were hybridized with gene-specific ³²P-end-labelled oligonucleotides (CrcZ: K3 (5'-GCT GGA GTC GTT ACG TGT

TG-3'); 5S rRNA: I26 (5'-CCC CAC ACT ACC ATC GGC GAT GCG TCG-3'). The hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

Determination of the Minimal Inhibitory Concentration (MIC) by Evaluator Strips (E-test)

Bacterial cultures were grown in BSM medium supplemented with 40 mM succinate to an OD₆₀₀ of 1.8. Then, 200 µl of cultures were plated on agar plates containing the respective media and MIC Evaluator strips for imipenem were applied with antibiotic concentrations of 0.002-32 µg/ml (Oxoid). The plates were incubated at 37°C and the MICs were determined by analyzing the growth inhibition zones. The MICs correspond to the lowest concentration of antibiotics that impeded growth.

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2 Supplementary Tables and Figures

2.1 Supplementary Tables

Supplementary Table S1. Strains and plasmids used in this study

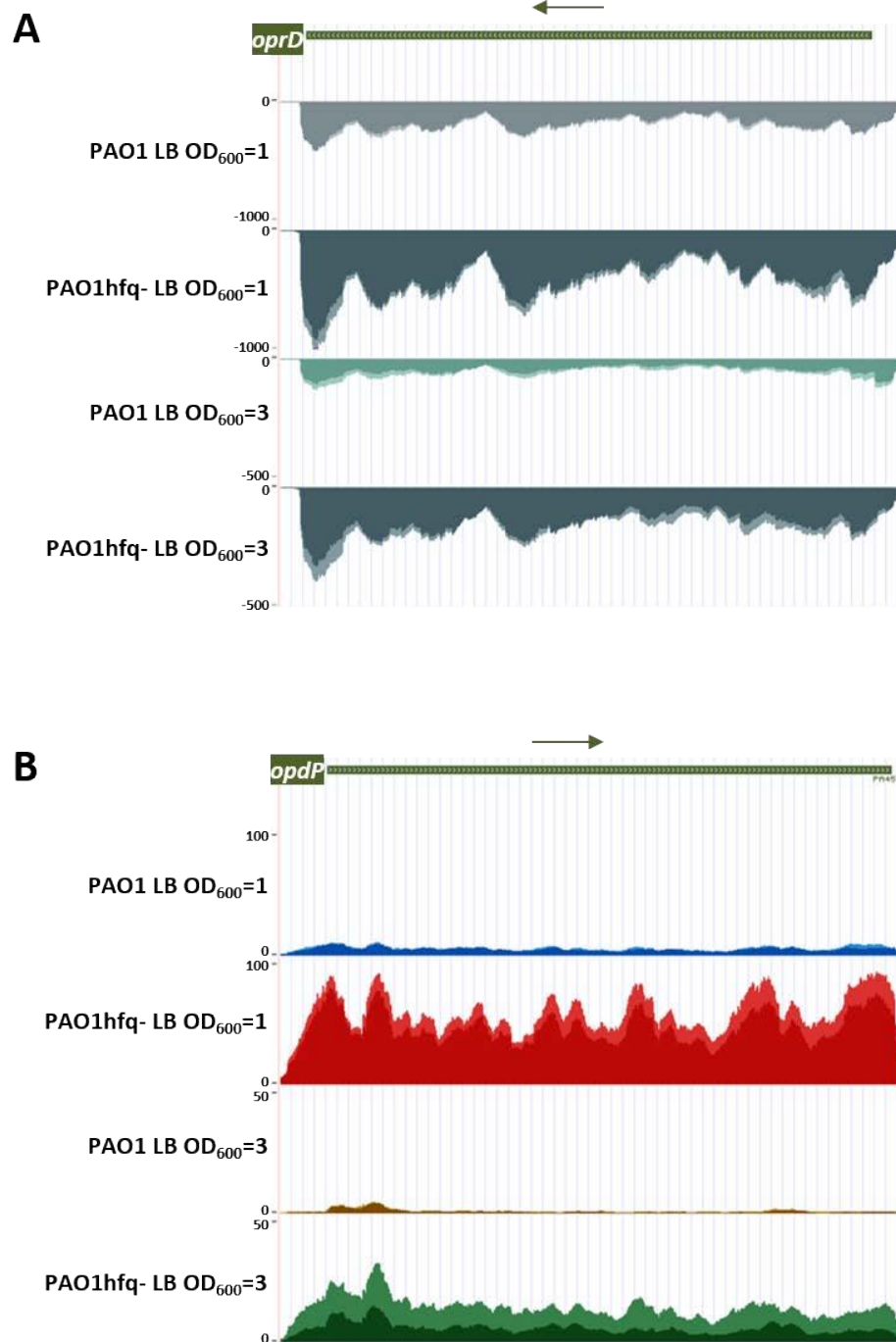
Strain/plasmid	Genotype/relevant features	Source/reference
<i>P. aeruginosa</i>		
PAO1		(Holloway et al., 1979)
PAO1 <i>hfq</i> -	<i>hfq::aadA</i> ; Sp/Sm ^R	(Sonnleitner et al., 2003)
PAO1Δ <i>hfq</i>	In frame deletion of <i>hfq</i>	(Sonnleitner et al., 2017)
PAOΔ <i>crc</i>	PAO6673, in frame deletion of <i>crc</i>	(Sonnleitner et al., 2009)
PAO1Δ <i>oprD</i>	In frame deletion of <i>occD1/oprD</i>	(Isabella et al., 2015)
PAO1Δ <i>opdP</i>	In frame deletion of <i>occD3/opdP</i>	(Isabella et al., 2015)
PAO1Δ <i>sr0161</i>	Deletion of <i>sr0161</i>	This study
PAO1Δ <i>ersA</i> Δ <i>sr0161</i>	Double deletion of <i>ersA</i> and <i>sr0161</i>	This study
PAO1Δ <i>hfq</i> Δ <i>ersA</i> Δ <i>sr0161</i>	Triple deletion of <i>hfq</i> , <i>ersA</i> and <i>sr0161</i>	This study
PAO1Δ <i>hfq</i> Δ <i>sr0161</i>	Double deletion of <i>hfq</i> and <i>sr0161</i>	This study
PA14		(Liberati et al., 2006)
PA14Δ <i>hfq</i>	In frame deletion of <i>hfq</i>	(Wurtzel et al., 2012)
Plasmids		
pME4510	Broad-host-range promoter-probe vector, Gm ^R	(Rist and Kertesz, 1998)
pME4510 <i>hfq</i> _{Flag}	pME4510 carrying PAO1 <i>hfq</i> fused to a Flag-tag encoding sequence under control of its authentic promoter	(Sonnleitner and Bläsi 2014)
pME4510 <i>crc</i> _{Flag}	pME4510 carrying PAO1 <i>crc</i> fused to a Flag-tag encoding sequence under control of its authentic promoter	(Sonnleitner et al., 2018)
pKH6	pJN105 derived vector for L-arabinose (L-ara) inducible expression of sRNAs, Gm ^R	(Han et al., 2016)
pKH6 <i>ersA</i>	L-ara inducible expression of <i>ersA</i>	(Zhang et al., 2017)
pKH6 <i>sr0161</i>	L-ara inducible expression of <i>sr0161</i>	(Zhang et al., 2017)
pME6032	<i>lacI</i> ^q -P _{tac} expression vector, Tc ^R	(Heeb et al., 2002)

pME6015	Cloning vector for translational <i>lacZ</i> fusions , Tc ^R	(Schnider-Keel et al., 2000; Kulkarni et al., 2014)
pME6015P _{tac}	Cloning vector for translational <i>lacZ</i> fusions under the control of a P _{tac} promoter	This study
pME6015P _{tac} <i>oprD</i> :: <i>lacZ</i>	pME6015P _{tac} containing a translational <i>oprD</i> :: <i>lacZ</i> fusion	This study
pME6015P _{tac} <i>opdP</i> :: <i>lacZ</i>	pME6015P _{tac} containing a translational <i>opdP</i> :: <i>lacZ</i> fusion	This study
pEXG2	ColE1 suicide vector; <i>mob sacB</i> Gm ^R	(Rietsch et al., 2005)
pEXG2- <i>sr0161</i>	pEXG2 with flanking regions to create an unmarked <i>sr0161</i> deletion	(Zhang et al., 2017)
pEXG2- <i>ersA</i>	pEXG2 with flanking regions to create an unmarked <i>ersA</i> deletion	(Zhang et al., 2017)
pMMB67HE	IncQ expression vector carrying an inducible P _{tac} promoter; Ap/Cb ^R	(Fürste et al., 1986)
pMMBcrcZ	pMMB67HE harboring <i>crcZ</i> under transcriptional control of P _{tac}	(Sonnleitner and Bläsi, 2014)

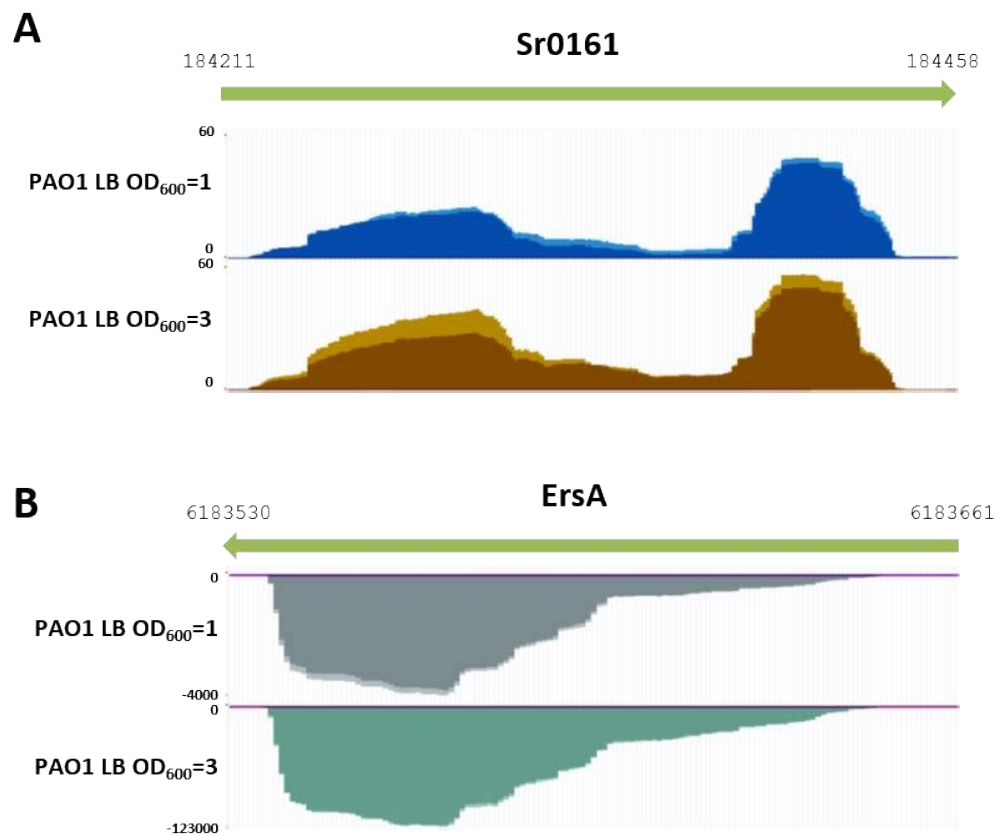
Supplementary Table S2 Transcript abundance of *oprD* and *opdP* in *P. aeruginosa* wild type and *hfq* deletion strains derived from different transcriptome analyses.

Comparison	Gene	Growth condition	Fold change (P _{adj} -value)	Base Mean wild type ^a	Base Mean mutant ^a	Reference
PA14Δ <i>hfq</i> vs PA14	<i>oprD</i>	SCFM, OD ₆₀₀ =2	5.0 (8.6 E-31)	10592	52700	Pusic et al., 2018
PA14Δ <i>hfq</i> vs PA14	<i>opdP</i>	SCFM, OD ₆₀₀ =2	20.7 (1.8 E-91)	443	9159	Pusic et al., 2018
PAO1 <i>hfq</i> - vs PAO1	<i>oprD</i>	BSM complex, OD ₆₀₀ =1,5	2.3 (6.0 E-8)	18487	42194	Sonnleitner et al., 2018
PAO1 <i>hfq</i> - vs PAO1	<i>opdP</i>	BSM complex, OD ₆₀₀ =1,5	22.6 (1.6 E-71)	107	2419	Sonnleitner et al., 2018
PAO1 <i>hfq</i> - vs PAO1	<i>oprD</i>	LB, OD ₆₀₀ =1	2.6 (4.8 E-41)	75093	19629	This study
PAO1 <i>hfq</i> - vs PAO1	<i>opdP</i>	LB, OD ₆₀₀ =1	11.6 (5.1 E-28)	1534	17803	This study
PAO1 <i>hfq</i> - vs PAO1	<i>oprD</i>	LB, OD ₆₀₀ =3	3.9 (7.8 E-18)	14580	56468	This study
PAO1 <i>hfq</i> - vs PAO1	<i>opdP</i>	LB, OD ₆₀₀ =3	13.9 (1.2 E-4)	177	2470	This study

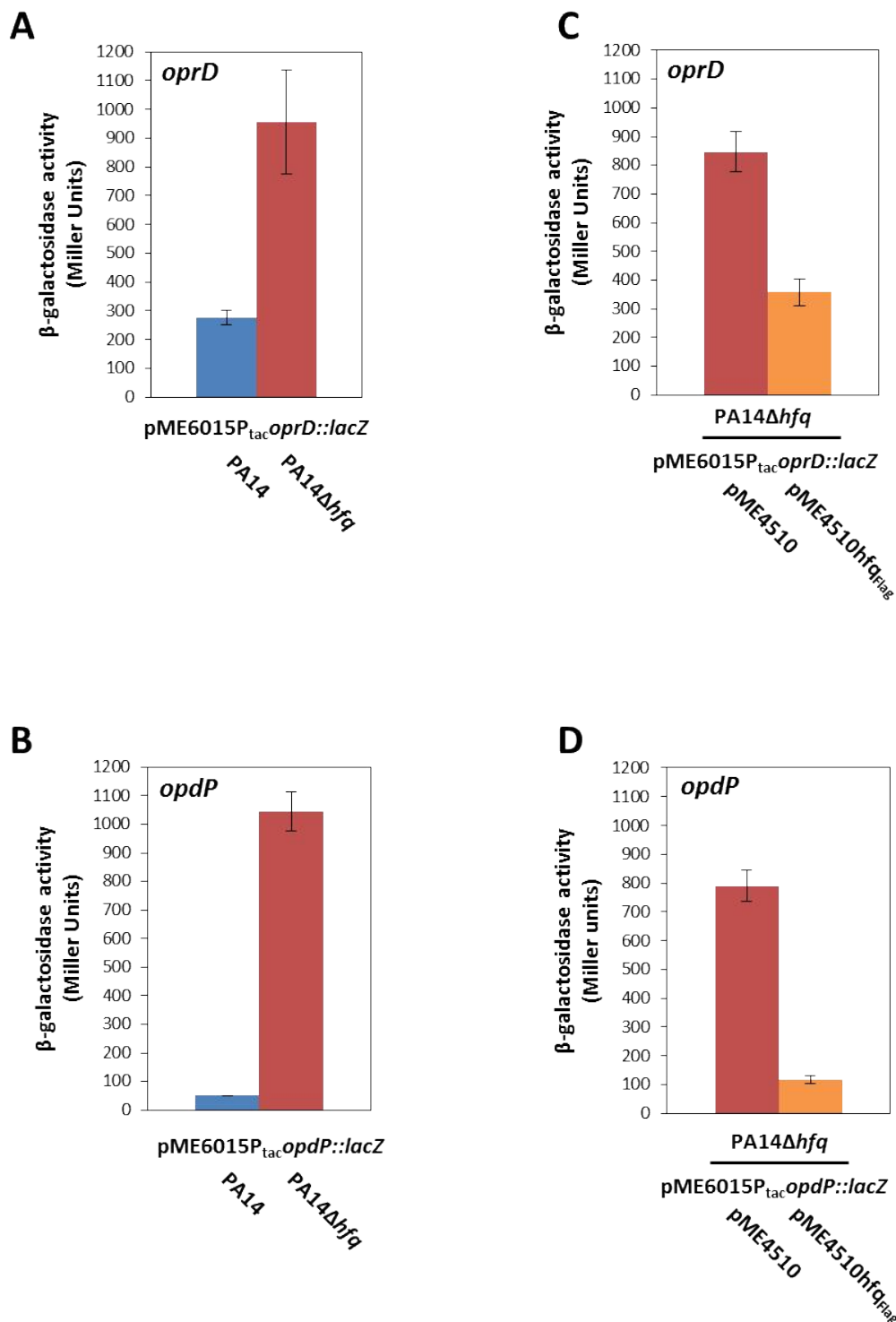
^a The results are given as base mean of two independent experiments



Supplementary Figure S1 Normalized read coverage visualized by the Genome Browser of the *oprD* (A) and *opdP* (B) transcripts after growth of PAO1 and PAO1hfq- in LB medium to an OD₆₀₀ of 1.0 (upper panels) and 3.0 (lower panels), respectively. The arrows indicate transcriptional directionality.

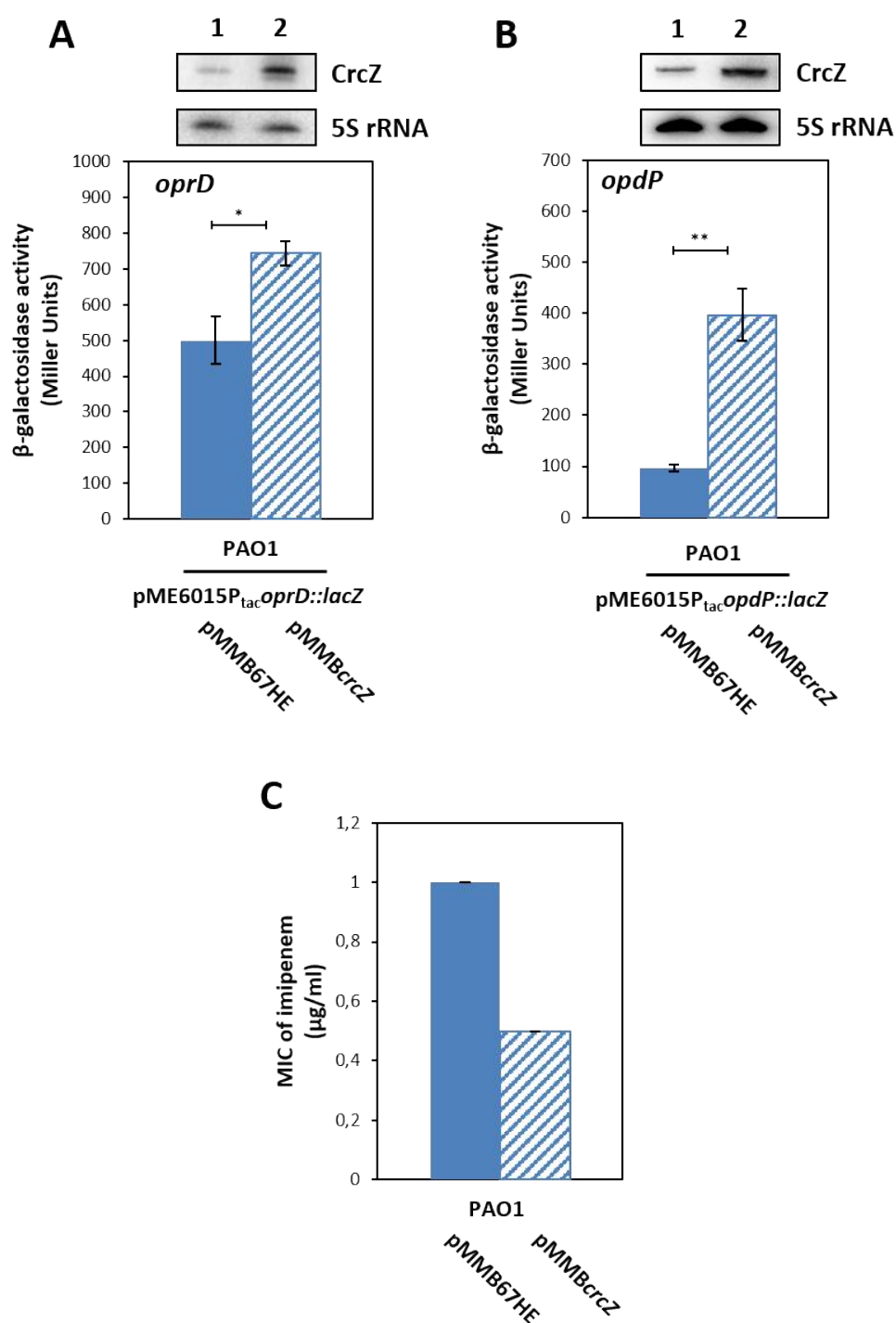


Supplementary Figure S2 Normalized read coverage visualized by the Genome Browser of the Sr0161 (A) and ErsA (B) sRNA transcripts after growth of PAO1 in LB medium to an OD₆₀₀ of 1.0 (upper panels) and 3.0 (lower panels), respectively. The arrows indicate transcriptional directionality.



Supplementary Figure S3 Hfq negatively regulates *oprD* and *opdP* translation in strain PA14. (**A**, **B**) The strains PA14 (blue bar) and PA14Δ*hfq* (red bar) harboring either plasmid pME6015P_{tac}*oprD::lacZ* (**A**) or pME6015P_{tac}*opdP::lacZ* (**B**) were grown in synthetic cystic fibrosis medium (SCFM) (Palmer et al., 2007) containing 100 μM FeSO₄ (Tata et al., 2016). Samples were withdrawn at an OD₆₀₀ of 2.0.

The bars represent the β -galactosidase values conferred by the *oprD::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*oprD::lacZ* (**A**) and by the *opdP::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*opdP::lacZ* (**B**), respectively. The error bars represent standard deviations from two independent experiments. The strains PA14 Δ *hfq*(pME4510) (red bar) and PA14 Δ *hfq*(pME4510*hfq*_{Flag}) (orange bar) harboring either plasmid pME6015P_{tac}*oprD::lacZ* (**C**) or pME6015P_{tac}*opdP::lacZ* (**D**) were grown in SCFM medium containing 100 μ M FeSO₄. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β -galactosidase values conferred by the *oprD::lacZ* translational fusion gene (**C**) and by the *opdP::lacZ* translational fusion gene (**D**), respectively, in the presence or absence of ectopic *hfq*_{Flag} expression. The error bars represent standard deviations from three independent experiments.



Supplementary Figure S4 Over-expression of *crcZ* results in increased *oprD/opdP* translation and susceptibility to imipenem. (**A**, **B**) The strains PAO1(pMMB67HE) (solid blue bar) and PAO1(pMMB*crcZ*) (striped blue bar) harboring either plasmid pME6015P_{tac}*oprD::lacZ* (**A**) or pME6015P_{tac}*opdP::lacZ* (**B**) were grown in BSM medium supplemented with 40 mM succinate. Samples were withdrawn at an OD₆₀₀ of 2.0. The bars represent the β-galactosidase values conferred

by the *oprD::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*oprD::lacZ* (**A**) and by the *opdP::lacZ* translational fusion gene encoded by plasmid pME6015P_{tac}*opdP::lacZ* (**B**), respectively, in the presence and absence of ectopic *crcZ* expression. The error bars represent standard deviations from three independent experiments. Top panels, CrcZ and 5SrRNA levels in strain PAO1(pMMB67HE) (lane 1) and PAO1(pMMB*crcZ*) (lane 2) harboring either plasmid pME6015P_{tac}*oprD::lacZ* (**A**) or pME6015P_{tac}*opdP::lacZ* (**B**). CrcZ and 5S rRNA levels were determined by Northern-blot analyses as described in Supplementary Text S1. (**C**) Minimal inhibitory concentration (MIC) of imipenem in strain PAO1(pMMB67HE) (solid blue bar) and PAO1(pMMB*crcZ*) (striped blue bar) grown in BSM medium supplemented with 40 mM succinate. The MIC was determined by E-test strips (Oxoid) with an antibiotic concentration range from 32–0.002 µg/ml imipenem. The experiment was performed with two biological replicates.