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„Requirement of Hfq for riboregulation and anaerobic growth of *Pseudomonas aeruginosa*“

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Konstantin Prindl, BSc, BEd

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1. Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a versatile, motile Gram-negative rod-shaped bacterium (Fig. 1) belonging to the phylum of Gammaproteobacteria. It is found in terrestrial and aquatic habitats, but is also able to colonize various plant and animal hosts (Stover *et al.,* 2000).

While *P. aeruginosa* preferentially grows under aerobic conditions, it is also able to thrive under anaerobic conditions using NO_3^-, NO_2^- or N_2O as terminal electron acceptors (Anzai *et al.,* 2000, Peix *et al.,* 2009, Sun Yoon *et al.,* 2002).

P. aeruginosa is known as a major opportunistic pathogen in humans, which rarely affects healthy people but has medical relevance as a nosocomial (hospital acquired) pathogen (Cross *et al.,* 1983). The pathogenicity is based on the metabolic versatility, the production of a diverse array of virulence factors and the formation of biofilms which is accompanied by an increased antibiotic tolerance (Balasubramanian *et al.,* 2013). The bacterium causes a variety of infections especially in patients suffering from urinary tract infections, severe burn wounds, AIDS, lung cancer, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (Bouza *et al.,* 2002, Van Delden & Iglewski 1998, Kerr & Snelling 2009, Manfredi *et al.,* 2000, Valderrey *et al.,* 2010). This diverse spectrum of infections requires a tightly regulated multifactorial virulence machinery. Approximately 10% of all open reading frames (ORFs) in *P. aeruginosa* encoding regulatory proteins which modulate its pathogenicity (Van Delden & Iglewski 1998, Mathee *et al.,* 2008). Cell-associated virulence factors of *P. aeruginosa* include flagella, adhesion factors, secretion systems, lipopolysaccharides and extracellular polysaccharides, while secreted virulence factors are comprised of exotoxins, exoenzymes, proteases, elastase, chitinase,

Fig. 1. Scanning electron microscopy image of *Pseudomonas aeruginosa* PA14 surrounded by extracellular matrix (Franklin *et al.,* 2011).

hemolysins, pyoverdine, pyocyanin and hydrogen cyanide (Van Delden & Iglewski 1998, Nicas & Iglewski 1985, Woods & Iglewski 1983).
Many of these virulence factors are indispensable to either establish an acute infection or required for the switch from an acute to a persistent infection (reviewed in Malhotra *et al.,* 2019).

1.2 Adapting to environmental stimuli: Quorum sensing

Quorum sensing (QS) is a bacterial cell-cell communication system, which allows Bacteria to coordinate group controlled behavior. It involves the production and secretion of a molecule termed autoinducer (AI), and subsequent recognition by a cognate response regulator. At low cell density the concentration of AI in the environment is low and the AI diffuses away. At high cell density, however, the local concentration of AI is high and is sensed by its cognate response regulator.

P. aeruginosa employs four quorum sensing systems, which regulate gene expression in a hierarchical manner; two LuxR and LuxI-type systems termed LasR/I and RhlR/I, as well as the *Pseudomonas* Quinolone Signal (PQS) and the IQS system (Fig. 2; Papenfort, & Bassler, 2016).

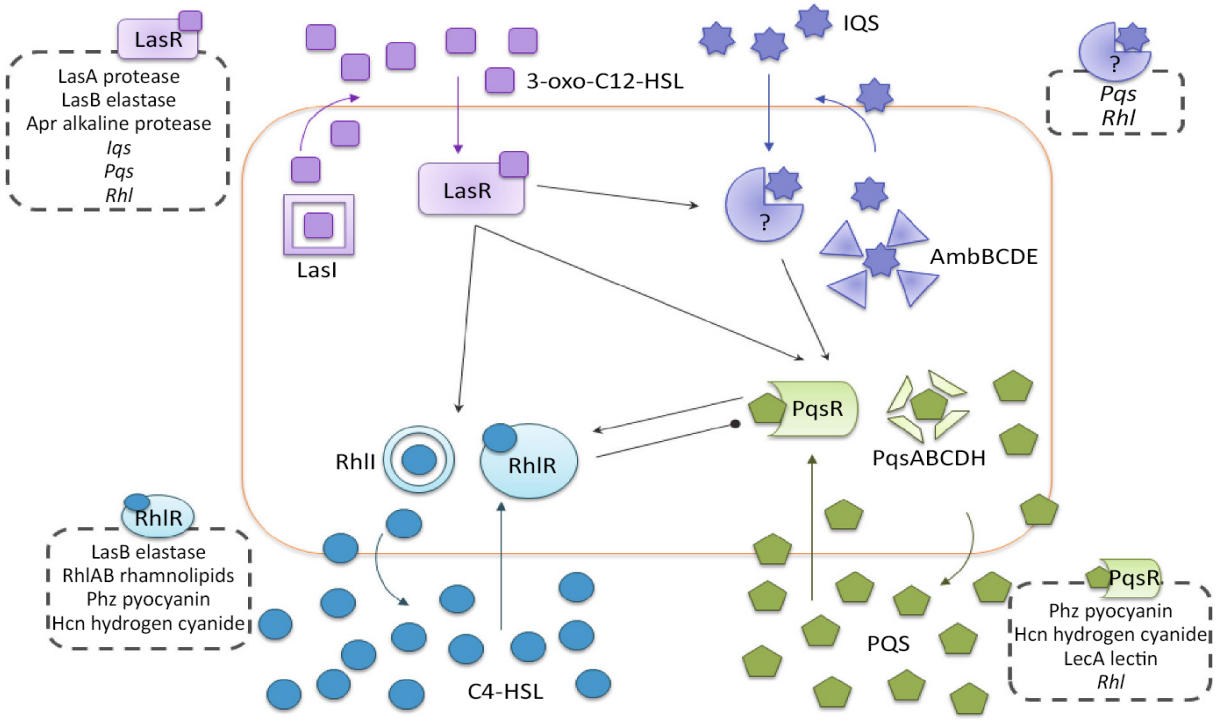

Fig. 2. *P. aeruginosa* utilizes four quorum sensing systems in a hierarchical manner (modified from Lee and Zhang 2014). The LasR/I quorum sensing system is at the top of the hierarchy. It activates and controls the *Iqs*, PQS and Rhl/R QS systems. Further down the hierarchy are the Rhl/R and PQS systems. When activated, RhlR activates transcription of many virulence factors, such as rhamnolipids, pyocyanin and hydrogen cyanide, while it represses PqsR. Activated PqsR on the other hand induces transcription of *rhlR* in addition to many virulence associated genes.

The LasR/I system acts at the top of the hierarchy; when LasR is bound to its autoinducer N-(3-oxododecanoyl)-homoserinelactone (3-oxo-C12-HSL), it multimerizes and binds to specific DNA sequences in the -40 region of the promoter containing a 5'-NNCT-$(N)_{12}$-AGNN-3' motif (Kiratisin *et al.,* 2002, Whiteley & Greenberg 2001, Whiteley *et al.,* 1999). This activates transcription of many downstream genes including the *lasI* synthase gene,

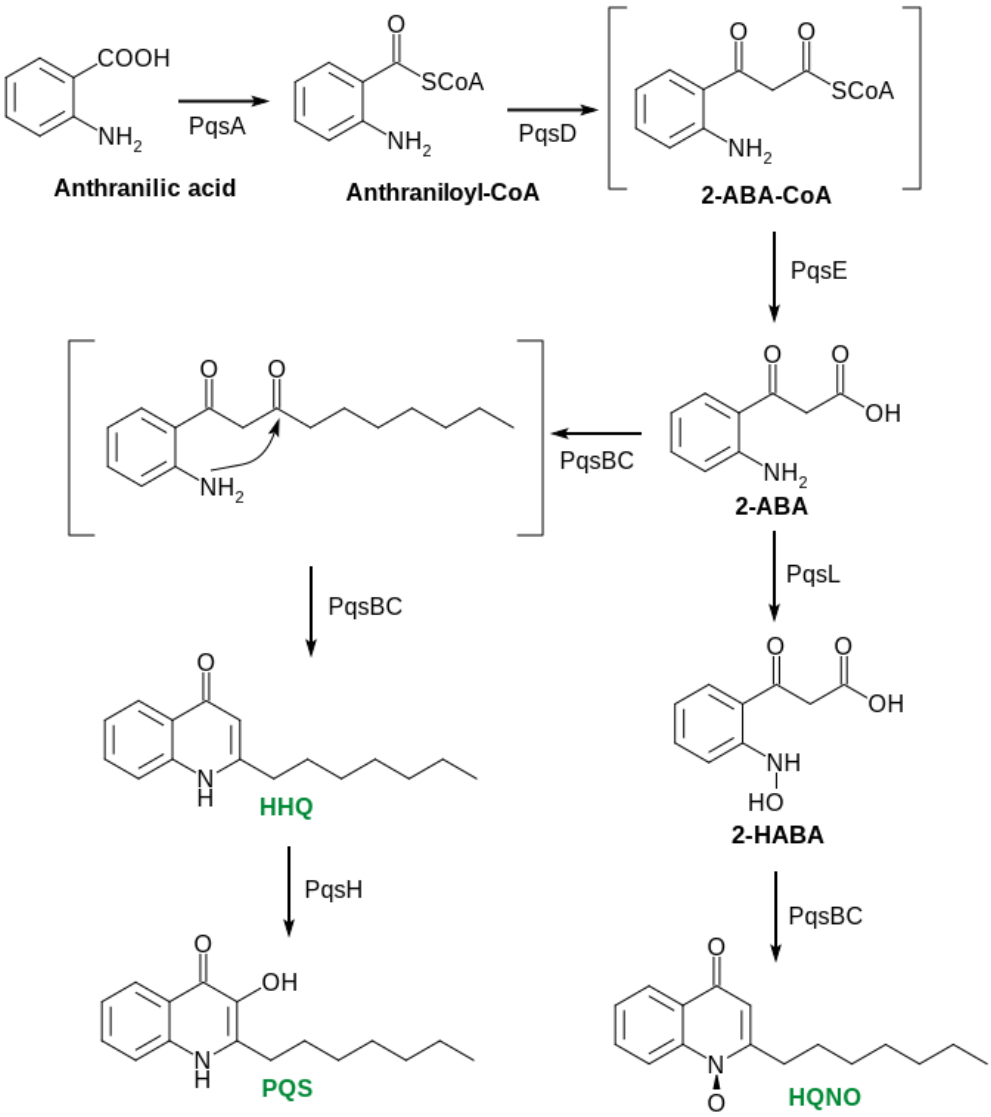

Fig. 3. PQS synthesis pathway. Anthranilic acid (AA) serves as the precursor of PQS synthesis. In the first step PqsA initiates the ligation of Coenzyme A (CoA) to yield anthranilolyl-CoA (AA-CoA). In the subsequent reaction PqsD catalyses the conversion of AA-CoA to 2-aminobenzoylacetyl-CoA (2-ABA-CoA). In the following reaction PqsE hydrolyses the ester bond yielding 2-animebenzoylacetate (2-ABA). 2-ABA can act as a substrate for either PqsL yielding 2-hydroxyanimobenoylacetate (2-HABA) or PqsBC which yields 2-heptyl-4-quinolone (HHQ). Finally PqsH catalyzes the formation of 2-heptyl-3-hydroxy-4-quinolone (PQS) from HHQ.

PqsBC shows higher substrate specificity for 2-HABA than 2-ABA, thereby synthesizing 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) from 2-HABA as a precursor.

which results in an autoinduction feed-forward loop (Latifi *et al.,* 1996, Pesci *et al.,* 1997, Seed *et al.,* 1995).

In addition, the expression of *rhlR* and *rhlI* genes (encoding the second QS system) (Ventre *et al.,* 2003, Winson *et al.,* 1995), the *pqsABCDH* and *pqsR* (activating the PQS system) are induced (Déziel *et al.,* 2004, Gallagher *et al.,* 2002, Xiao *et al.,* 2006a). RhlI synthesizes N-butyryl-homoserine lactone (C4-HSL), which is recognized by the second LuxR-type regulator RhlR. RhlR is able to bind to similar recognition motifs as LasR, thereby partly overlapping the regulon of LasR (Schuster & Greenberg 2007, Whiteley & Greenberg 2001, Whiteley *et al.,* 1999). Binding activates transcription of downstream genes, including *rhlI* and *rhlR* creating the second feed-forward loop (Ventre *et al.,* 2003, Winson *et al.,* 1995). Transcriptomic studies revealed that some genes are activated preferentially by LasR, others by RhlR and several equally well by both regulators (Schuster & Greenberg 2006, Schuster & Lostroh 2003). The PqsR-PQS complex in turn activates transcription of itself and the genes required for PQS biosynthesis *pqsABCDE* and *pqsH* (Gallagher *et al.,* 2002, Wade *et al.,* 2005, Xiao *et al.,* 2006b), and feeds information back to the *rhl* system by activating *rhlRI* transcription (McKnight *et al.,* 2000, Pesci *et al.,* 1999). Interestingly, RhlR represses the expression of *pqsR* and *pqsABCD*, which is suggested to maintain the ratio of C4-HSL to PQS (Cao *et al.,* 2001).

The quinolone 2-heptyl-3-hydroxy-4-quinolone (PQS) is synthesized from anthranilate (AA) as a precursor by the *pqsABCD*, *pqsE*, *phnAB* and *pqsH* gene products (Fig. 3; Gallagher *et al.,* 2002). PqsA is an anthranilate-coenzyme A ligase, initiating the first step in the biosynthesis of PQS (Coleman *et al.,* 2008, Gallagher *et al.,* 2002). PqsD catalyses the synthesis of 2-aminobenzoylacetate from anthraninoyl-CoA (Dulcey *et al.,* 2013), whereas PqsE is a thioesterase facilitating the formation of 2-aminobenzoylacetate (2-ABA; Drees & Fetzner 2015). PqsBC facilitates the subsequent reaction from 2-ABA to 2-heptyl-4-quinolone (HHQ) (Déziel *et al.,* 2004, Gallagher *et al.,* 2002), an important precursor of PQS. However, 2-ABA may also act as a substrate for PqsL forming 2-hydroxyaminobenzoylacetate (2-HABA), an important precursor for the subsequent formation of 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) by PqsBC (Drees *et al.,* 2018). PQS is the most potent activator for PqsR, it has been suggested that the intermediates HHQ, DNQ and HQNO are able to facilitate PQS signaling to some extent (Wade *et al.,* 2005, Xiao *et al.,* 2006a). PQS is finally synthesized from HHQ by the PqsH enzyme (Déziel *et al.,* 2004, Dubern & Diggle 2008, Gallagher *et al.,* 2002). Virulence factors controlled by the PQS system include pyocyanin, PA-IL lectin and rhamnolipids which require functional PQS signaling (Cao *et al.,* 2001, Diggle *et al.,* 2003, Rahme *et al.,* 1997, 2000). While most virulence genes are controlled by the *rhl* system, the PQS system is indispensible for expression of full virulence towards *C. elegans*, *Drosophila melanogaster* and mice (Cao *et al.,* 2001, Gallagher *et al.,* 2002, Lau *et al.,* 2004).

The fourth QS system, IQS, integrates environmental cues into the QS circuitry. (Lee *et al.,* 2013). While it is tightly controlled by the LasI/R system in rich medium, it was shown to activate the QS genes in response to phosphate limitation, and is able to

overcome *lasR* mutations in clinical isolates. This activation is achieved by the PhoB histidine kinase. 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) was suggested to be produced by the gene products encoded by the *ambBCDE* operon. The AmbBCDA gene products were shown to be responsible for synthesis of the L-2-amino-4-methoxy-trans-3-butenoic acid instead of IQS (Murcia *et al.,* 2015). It was shown, that the IQS is able to complement a *lasR* mutation by activating transcription of genes of both the Rhl/R and the PQS systems (Lee *et al.,* 2013).

1.3. Adaptation to starvation: the bacterial stringent response

An additional system employed by Bacteria to respond to environmental signals is the stringent response and the metabolism of the signaling molecules that govern it; ppGpp and pppGpp. (p)ppGpp was identified as key inhibitor of RNA synthesis during amino acid starvation (Cashel & Gallant 1969), during which uncharged tRNAs activate synthesis of (p)ppGpp by RelA (Wendrich *et al.,* 2002). Later, the function of (p)ppGpp was expanded to respond to different stress conditions and to regulate various cellular functions (Kriel *et al.,* 2012).

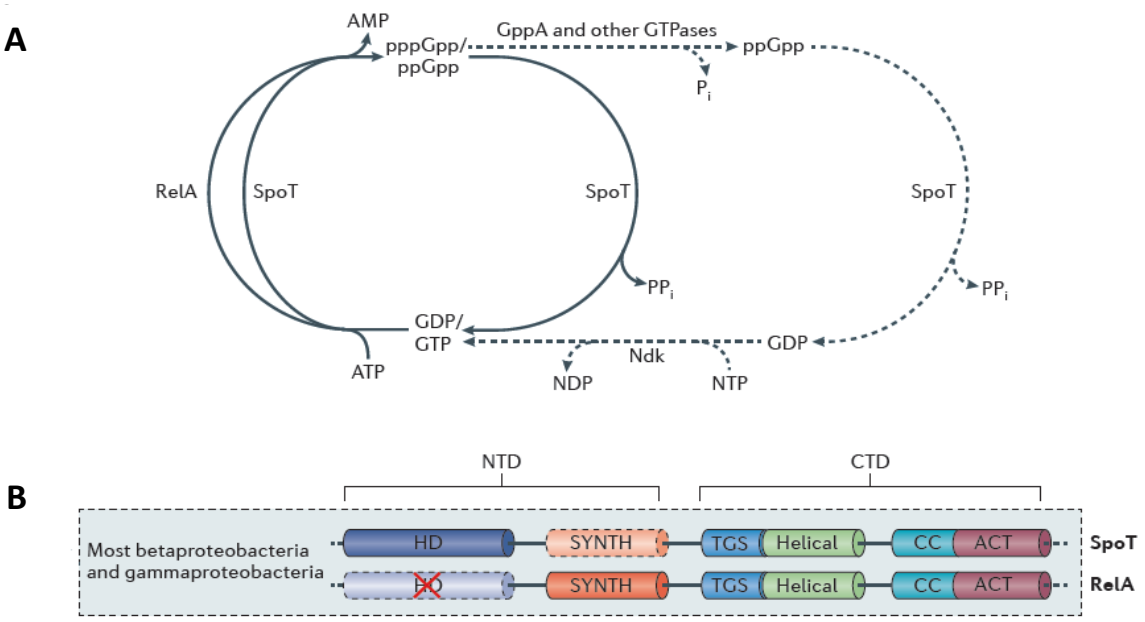

Fig. 4. (p)ppGpp metabolism regulated by RelA and SpoT (Hauryliuk *et al.,* 2015). (**A**) Schematic representation of the (p)ppGpp metabolism in *E. coli*. RelA and SpoT synthesize pppGpp from GTP and ppGpp from GDP. Interconversion of pppGpp to ppGpp is catalysed by GppA . Degradation od (p)ppGpp is catalysed by SpoT generation GTP or GDP. (**B**) The domains of SpoT and RelA as they occur in most gamma- and betaproteobacteria. In the case of SpoT the (p)ppGpp synthesis (SYNTH) domain exhibits only weak enzymatic activity, while the hydrolytic activity of the RelA hydrolytic domain (HD) is completely absent. Both of the domains are located in the N-terminal domain of SpoT/RelA.

(p)ppGpp metabolism is meditated by the RelA and SpoT enzymes; RelA and SpoT synthesize pppGpp and ppGpp from ATP and GTP or GDP respectively (Fig. 4A; Atkinson, *et al.,*, 2011, Potrykus, & Cashel, 2008) generating AMP as a byproduct. The structures of RelA bound to the ribosome (Arenz *et al.,* 2016, Brown *et al.,* 2016, Loveland *et al.,* 2016) suggest, that the C-terminal autoregulatory domain of RelA interacts directly with uncharged tRNAs in the ribosomal A-site and induces the (p)ppGpp synthase activity. In charged tRNAs this interaction is prevented by the amino acid, thereby inhibiting the ppGpp synthase activity (Gourse *et al.,* 2018). SpoT shows only weak synthetic activity (An *et al.,* 1979, Xiao *et al.,* 1991) but strong hydrolytic activity (i.e. conversion of (p)ppGpp to GTP/GDP), while RelA has no hydrolytic activity but strong synthetic activity. The hydrolytic function of SpoT is indispensable for the regulation and balancing of (p)ppGpp levels in the presence of RelA and the disruption of the *spoT* gene in *E. coli* is lethal (Xiao *et al.,* 1991). In accordance, both a *ΔrelA* mutant and the Δ*spoT*Δ*relA* double mutant are viable (Xiao *et al.,* 1991).

In *E. coli,* (p)ppGpp targets the RNA polymerase (RNAP) directly and binds as an allosteric regulator (Artsimovitch *et al.,* 2004) to the β and β' subunits of the RNAP holoenzyme (Artsimovitch *et al.,* 2004).

The DksA protein was also found to bind directly to the RNAP (Paul *et al.,* 2004, Perederina *et al.,* 2004). In vitro studies found, that transcription including both DksA and ppGpp resulted in a decreased transcription from rRNA promoters, while stimulating transcription of amino acid and transport promoters (Paul *et al.,* 2004). These promoters have previously been identified as stimulated by ppGpp *in vivo* (Paul *et al.,* 2005).

In *P. aeruginosa* (p)ppGpp and the stringent response plays a role in virulence (Erickson *et al.,* 2004, Vogt *et al.,* 2011), resistance to UV radiation (Pezzoni *et al.,* 2012) and under oxidative stress (Khakimova *et al.,* 2013). It was reported that the stringent response mediates antibiotic tolerance in nutrient-starved cell and biofilm conditions (Nguyen *et al.,* 2013). A Δ*spoT*Δ*relA* mutant was shown to be highly sensitive to multiple antibiotics (Nguyen *et al.,* 2013). Additionally a link between QS and the stringent response was previously reported by several groups (Van Delden *et al.,* 2011, Schafhauser *et al.,* 2014). A (p)ppGpp deficient strain was reported to exhibit 2 to 14-fold higher levels of HHQ and PQS than the wild type, which could be explained by increased expression of *pqsA*, *pqsH* and *pqsR* (Schafhauser *et al.,* 2014). In addition, (p)ppGpp was reported to be required for the full activation of both the *las* and *rhl* QS systems (Schafhauser *et al.,* 2014).

1.4. Transcriptional switching in bacteria: alternative sigma factors

An additional mechanism to regulate gene expression in response to changes in the environment is conferred by the use of alternative sigma factors. Initiation of transcription is performed by the multi-subunit RNA polymerase (RNAP) which consists of 5 subunits (α_2, β, β' and ω) and a dissociable σ factor (Burgess *et al.,* 1969, Travers & Burgess 1969)

binding to the core RNAP (Gruber & Gross 2003). The σ proteins are necessary for recognition of promoter elements (Gross *et al.,* 1998) and for melting the DNA to form the open promoter complex (Fig. 5).

Sigma factors can be divided into two classes, σ^{70}-like and σ^{54}-like. Genetic screens in *P. aeruginosa* identified 24 putative sigma factors, of which 19 belong to the extracytoplasmic family (ECF; reviewed in Potvin *et al.,* 2007). *P. aeruginosa* has a major σ^{70} factor encoded by the gene *rpoD.* σ^{70} recognizes the majority of promoters and controls the expression of housekeeping genes (Fujita *et al.,* 1994, Lonetto *et al.,* 1992). Of the σ^{70}-like class, RpoH (σ^{32}), FliA (RpoF, σ^{28}), RpoS (σ^{38}) as well as the ECF AlgU (RpoE, σ^{22}), PvdS (Cunliffe *et al.,* 1995, Leoni *et al.,* 1996, Wilson & Lamont 2000) and SigX have been studied (Brinkman *et al.,* 1999, Fléchard *et al.,* 2018). *P. aeruginosa* encodes one σ^{54} homologue, RpoN (formally NtrA) that was first identified in *E. coli* (Hirschman *et al.,* 1985, Hunt & Magasanik 1985). Promoters that depend on RpoN contain a highly conserved -24 (GC) / -12 (GG) motif, with a 10 nucleotide spacer in between (T**GG**CAC-N5-TT**GC**; Shingler 1996).

Initial observations on RpoN were linked solely to nitrogen assimilation, however, later RpoN has been linked to important roles in regulation of motility, transport of nutrients, formation of pili, cell adhesion and cell-to-cell signaling (Boucher *et al.,* 2000, Dasgupta *et al.,* 2003, Heurlier *et al.,* 2003, Ishimoto & Lory 1989, Ramphal *et al.,* 1991,

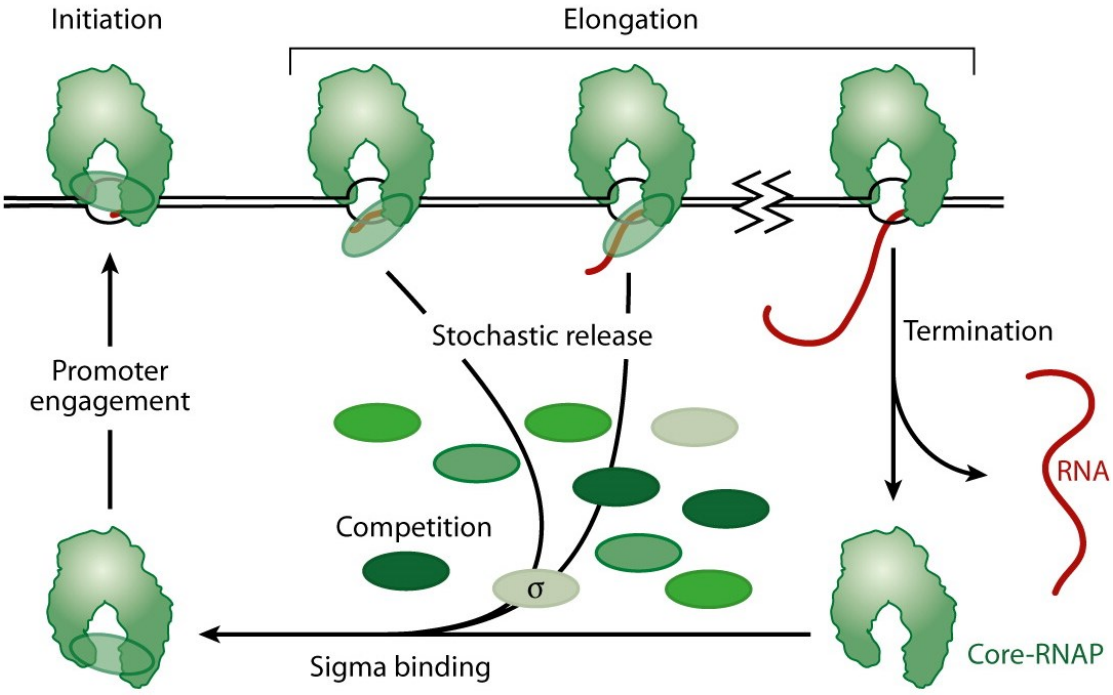

Fig. 5. The sigma cycle (Österberg *et al.,* 2011). Schematic of the transcription cycle in which sigma factors compete for association with the core-RNAP. This allows the holoenzyme to recognize various promoters. Sigma bound RNAP binds promoter DNA to form the initial closed complex. Through sigma-assisted formation of a number of instable intermediates, eventually the open complex is formed which is able to initiate transcription. These steps are reversible until the elongation complex escapes the promoter.

1996, Thompson *et al.,* 2003). Transcriptome studies revealed the RpoN regulon to be the largest among all alternative sigma factors, encompassing 680 genes (Schulz *et al.,* 2015). However, RpoN is fundamentally different from σ^{70}-like sigma factors. In contrast to the σ^{70} bound holoenzyme ($E\sigma^{70}$), which may transit from the initial closed complex to the transcriptionally active open complex without further regulatory factors, the $E\sigma^{54}$ complex is kinetically as well as thermodynamically stable (Buck *et al.,* 2000). Additionally, $E\sigma^{54}$ bears resemblance to eukaryotic RNA Pol II (Wang *et al.,* 1992). This complex is not able to melt DNA on its own, but transition to the open complex is dependent on the interaction with and nucleotide hydrolysis by enhancer binding proteins (EBPs) (Buck *et al.,* 2000). This allows almost instant expression of RpoN regulated genes in response to environmental changes (Genschik *et al.,* 1998, Lenz *et al.,* 1997, Weiner *et al.,* 1991). Several EBPs in *P. aeruginosa* (often part of two component systems) are known to activate RpoN dependent promoters: AlgB is required for the regulation of alginate production and mucoid colony morphology (Leech *et al.,* 2008, Wozniak & Ohman 1991), FleQ is involved in regulation of the biosynthesis of flagellin and flagella (Dasgupta *et al.,* 2003), PhhR is required for metabolism of L-phenylalanine and L-tyrosine (Song & Jensen 1996), PilR is the activator of genes involved in pilin formation (Ishimoto & Lory 1992), CbrB controls the utilization of different carbon-sources (Nishijyo *et al.,* 2001) and together with NtrC the utilization of N-sources (Li & Lu 2007).

The utilization of alternative sigma factors is also regulated by the signaling molecule (p)ppGpp (Jishage *et al.,* 2002). Destabilization of the $E\sigma^{70}$ holoenzyme by (p)ppGpp at rRNA promoters consequently causes massive down-regulation of these promoters and increases the availability of the core RNAP for binding to alternative sigma factors (Laurie *et al.,* 2003).

1.5. The global transcriptional regulator Anr

In *Pseudomonas* a major transcriptional regulator is Anr which controls anaerobic metabolism by activation of target genes. Anr is the oxygen-sensing regulatory protein homologue to the *E. coli* Fnr (Ray & Williams 1997, Sawers 1991, Zimmermann *et al.,* 1991). Anr, like Fnr contains four essential conserved cysteine residues (Green *et al.,* 1993, Spiro 1994) forming an iron-sulfur cluster (Fe-S cluster; Khoroshilova *et al.,* 1995). When concentration of oxygen is low, this Fe-S cluster enables Fnr/Anr to dimerize and to form the active transcriptional regulator (Lazazzera *et al.,* 1996), which is able to bind to 5'-TTGATNNNNATCAA-3' motifs (Winteler & Haas 1996). However, if the concentration of oxygen is high, Fe^{2+} is oxidized, leading to an increase of the monomeric form and to a decreased DNA binding affinity (Lazazzera *et al.,* 1996). In its active form Anr is involved in the induction of various regulons including those for arginine and pyruvate fermentation(Schreiber *et al.,* 2006), redox-state maintenance, fimbria and denitrification (Arai 2011, Arai *et al.,* 1995, 1997). Recently Anr was shown to induce expression of the oxygen independent class II ribonucleotide reductase (Crespo *et al.,* 2016), responsible for supplying precursor nucleotides for DNA synthesis.

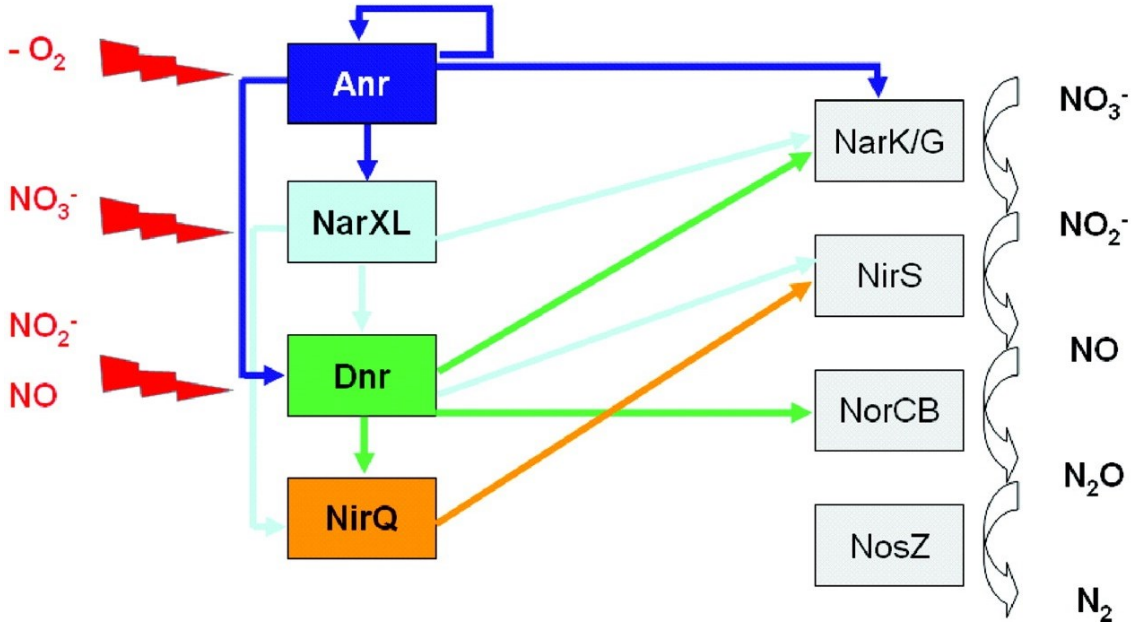

Fig. 6. The regulatory network for anaerobic respiration in *P. aeruginosa* and its regulation by Anr (Schreiber *et al.,* 2007). The major signal for induction of denitrification is a low oxygen concentration. Anr increases the transcription of the *narXL* operon, encoding a two-component system that responds to the presence of NO_3. Anr and NarL cooperatively activate transcription of the *dnr* gene, encoding an Fnr-like regulator responding to NO (Arai *et al.,* 1997). The final regulatory system NirQ requires both Dnr and NarL for its activation (Arai *et al.,* 1994).

During anaerobic growth, Anr enhances transcription of the *narXL* operon (Fig. 6; Schreiber, *et al.,*, 2007). This encodes a two-component regulatory system, which responds to the presence of nitrate. Both Anr and NarL cooperatively activate the transcription of *dnr*, coding for the third regulator (Arai *et al.,* 1997), which was shown to respond to NO in *P. stutzeri* (Vollack & Zumft 2001). Transcriptional activation of the fourth regulatory system is in turn activated by both NarL and Dnr (Arai *et al.,* 1994). Under low oxygen concentrations and abundance of nitrate, Anr and NarL activate together with IHF transcription of the *narK$_1$K$_2$GHJI* operon encoding nitrate/nitrite transporters as well as the structural genes for the nitrite reductase (Schreiber *et al.,* 2007). The expression of the gene encoding nitrite reductase *nirS* is induced by nitrate and nitrite (Arai *et al.,* 1991), and is most likely indirectly activated by NarL *via* Dnr (Fig. 6; Schreiber, *et al.,*, 2007). A similar pattern was observed for the transcription of *norC*, encoding a nitric oxide reductase cytochrome *c* subunit. Induction of *norC* was shown to be strongly dependent on nitrite, Anr and Dnr (Fig. 6; Arai, *et al.,*, 1995). Schreiber *et al.,* (2007) described an indirect activation of *norC via* NarL and Dnr.

Anr is not only involved in anaerobic respiration. It additionally regulates the expression of various other functions in *P. aeruginosa*, such as the induction of quorum sensing (Fig. 7; Sonnleitner, *et al.,*, 2011), the regulation of heme biosynthesis (Hammond *et al.,* 2015, Rompf *et al.,* 1998), the production of hydrogen cyanide and pyochelin biosynthesis (Hammond *et al.,* 2015). Anr has been shown to activate transcription of the

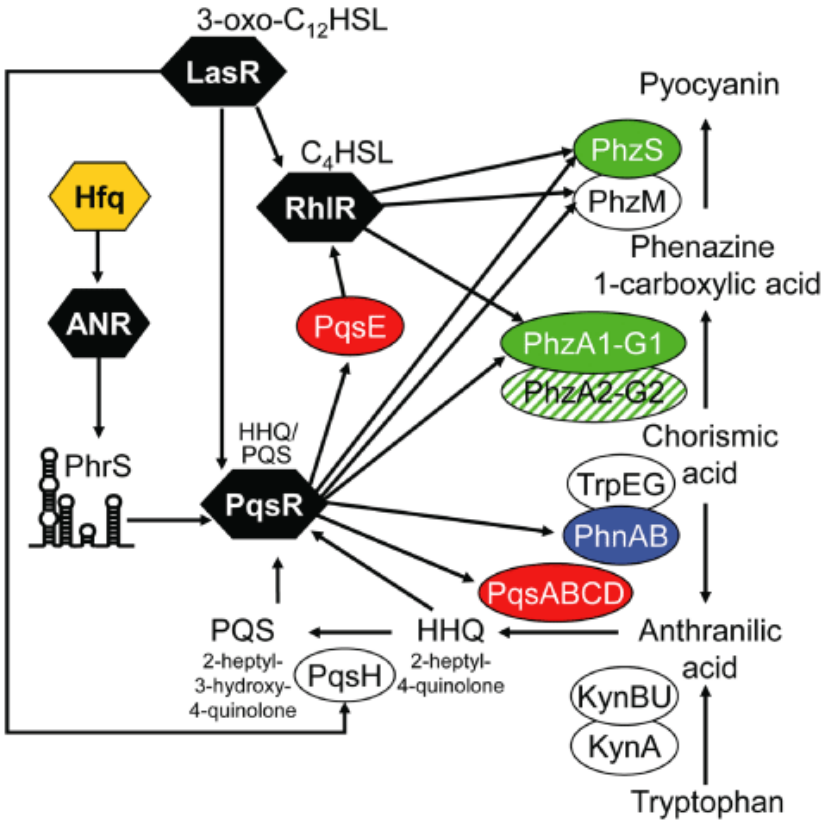

Fig. 7. Overview of PQS and pyocyanin synthesis and its regulation by the small RNA PhrS (Sonnleitner *et al.,* 2011). Transcription of PhrS is up-regulated when oxygen is limiting. This requires the anaerobic regulator Anr. PqsR positively regulates *phnAB* and the *pqsABCD* genes required for synthesis of PQS, as well as *pqsE*. PqsR is further essential for the transcription of the *phzA1-G1* operon as well as for the *phzS* and *phzM* genes, which are involved in pyocyanin synthesis. Upon over-expression of PhrS the expression of genes shown in green, red and blue was up-regulated (Sonnleitner *et al.,* 2011).

small RNA PhrS (Sonnleitner *et al.,* 2011). PhrS was shown to up-regulate genes required for PQS and pyocyanin synthesis which are under positive control of the transcriptional regulator PqsR (Déziel *et al.,* 2005, Sonnleitner *et al.,* 2011), providing a link between quorum sensing and anaerobic metabolism. Furthermore Anr is involved in iron acquisition and storage as well as in HAQ production (Hammond *et al.,* 2015). This might be a direct and indirect consequence of increased expression of the genes coding for the degradation of aromatic compounds, *antABC*, *catBCA* and *antR* (Hammond *et al.,* 2015). Interestingly, *anr* expression is stimulated by the RNA chaperone Hfq (Sonnleitner *et al.,* 2006, 2011), although the mechanism is currently unclear.

1.6. Hfq as global post-transcriptional regulator

A major post-transcriptional regulator is the RNA chaperone Hfq. Hfq was first discovered in *E. coli* to be required for replication of the phage Qβ (Carmichael *et al.,* 1975, Franze de Fernandez *et al.,* 1968). It is a hexameric RNA binding protein belonging to the family of Sm/LSm-like proteins (Moll *et al.,* 2003b, Moller *et al.,* 2002) that is present in all three domains of life (Achsel *et al.,* 2001, Salgado-Garrido *et al.,* 1999).

The crystal structure of both *E. coli* as well as *P. aeruginosa* Hfq revealed a donut shaped homo-hexameric protein (Nikulin *et al.,* 2005, Sauter *et al.,* 2003). The conserved core of Hfq is composed of one α-helix and five β-strands (Fig. 6A). The Sm1 motif consists of the α-helix and the first three β-strands, whereas the Sm2 motif is composed of the remaining two β-strands (Fig. 8A). Six of these protomers compose the Hfq homo-hexamer. Hfq possesses several RNA binding surfaces for the interaction with RNAs, with each surface binding to distinct RNA motifs (Vogel & Luisi 2011). The surface exposing the N-terminal α-helices is termed proximal side, while the opposite is termed the distal side. In a complex with poly(A) single stranded RNA the distal face of the *E. coli* Hfq protomer was found to contact three RNA nucleotides (Fig. 8B, bottom). An AR(A)N motif (A, represents an adenine; R, represents a purine base; N can be any base) was found to bind preferentially to the RNA binding pockets situated at the distal side (Link *et al.,* 2009, Mikulecky *et al.,* 2004). In a complex formed with *S. aureus* Hfq, single stranded poly(U) heptamer RNA was shown to bind around the central pore of the proximal face with each protomer contacting a single nucleotide (Fig. 8B, top; Schumacher, *et al.,*, 2002). In accordance with this, the distal RNA binding domain was shown to bind to mRNAs, while

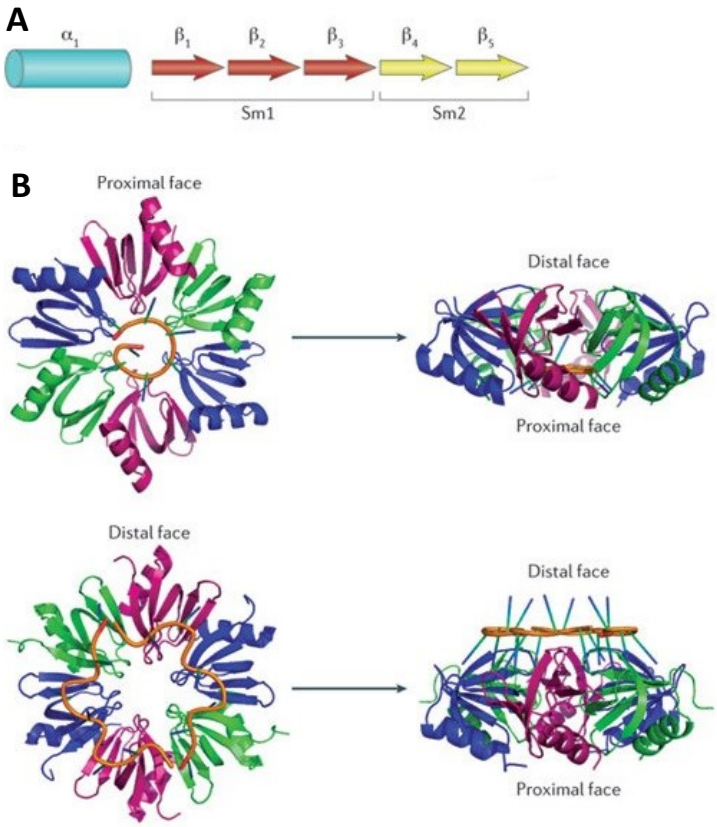

Fig. 8. Structure of the Sm-like RNA chaperone Hfq (modified from Vogel & Luisi 2011). (**A**) The conserved core of the protein consists of one α-helix and five β-strands. Of these 5 β-strands β_1 - β_3 make up the highly conserved Sm1 motif, while strands β_4 - β_5 form the Sm2 motif. (**B**) The interaction of RNA with the proximal binding face of Hfq (top) is shown. Each protomer of Hfq is shown to contact a single nucleotide of the RNA. Interaction of the RNA with the distal binding surface of Hfq (bottom). Here, each protomer contacts three nucleotides.

the proximal RNA binding face was shown to preferentially bind sRNAs. The simultaneous binding of both sRNAs and mRNAs to Hfq may facilitate their interaction (Vogel & Luisi 2011). In addition, the number of arginine residues in basic patches on the rim of the Hfq protein have been shown to promote selectivity of base pairing and to increase annealing (Panja *et al.,* 2013, 2015, Zheng *et al.,* 2016). Studies have shown that the flexible C-termini of Hfq are not needed for RNA annealing. They rather seem to promote RNA competition by displacing unstable RNA complexes (Santiago-Frangos & Woodson 2018, Santiago-Frangos *et al.,* 2016).

In *E. coli* expression of the *hfq* gene is tightly controlled, with levels increasing during growth (Azam *et al.,* 1999, Kajitani *et al.,* 1994, Tsui *et al.,* 1997). However, in *P. aeruginosa* the levels of Hfq are quite constant with ≈2200 hexamers per cell (Sonnleitner & Bläsi 2014). Although Hfq from PAO1 is lacking some features of the *E. coli* homologue, it is able to complement an *E. coli hfq*$^{-}$ strain in terms of Qβ replication and *rpoS* regulation (Sonnleitner *et al.,* 2002), demonstrating its highly conserved function. Deletion of *hfq* in *P. aeruginosa* leads to a pleiotropic phenotype, including an attenuated virulence, reduced oxidative stress tolerance and impaired motility (Sonnleitner *et al.,* 2003).

A pivotal function of Hfq entails the ability to facilitate base-pairing between two RNAs (*e.g.* between a small regulatory RNA (sRNA) and its target mRNA), by increasing the rate of complex formation between RNA and Hfq (Afonyushkin *et al.,* 2005, Fender *et al.,* 2010, Holmqvist *et al.,* 2010, Kawamoto *et al.,* 2006, Moller *et al.,* 2002, Zhang *et al.,* 2002). In accordance with its RNA chaperone function, Hfq is able to induce conformational changes in the secondary structure of RNAs upon binding (Geissmann & Touati 2004, Zhang *et al.,* 2002). Fast initial contact with RNAs is essential as the time frame from sRNA expression to a significant regulation of target genes is short (1-2 min) (Fender *et al.,* 2010, Massé *et al.,* 2003). The active cycling model explains how the formation of stable Hfq-RNA complexes can be reconciled with the rapid exchange of

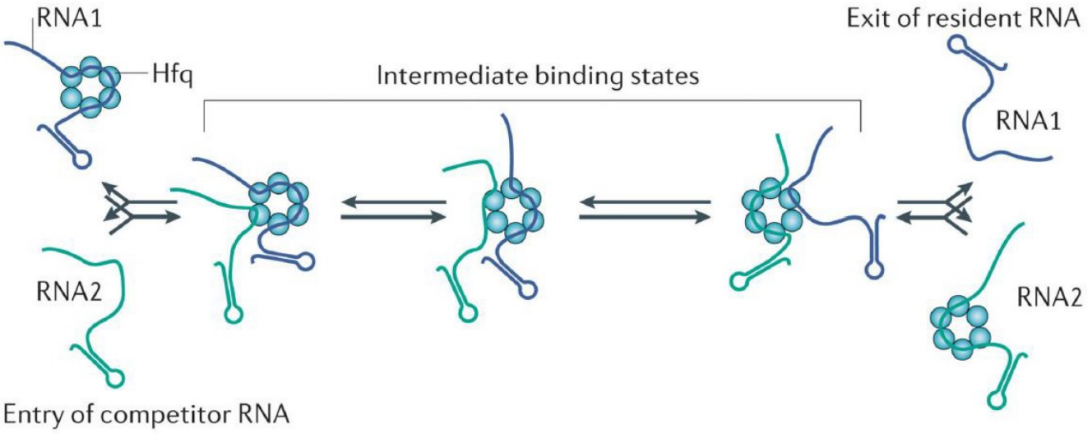

Fig. 9. The model of RNA exchange on Hfq (Vogel & Luisi 2011). A competitor RNA (RNA2) may transiently bind to a free Hfq binding site gradually replacing the resident RNA (RNA1) in a series of intermediate reversible binding steps. Rapid dissociation of either RNA is thought to occur from a one-protomer-bound state.

sRNAs on Hfq *in vivo* (Fig. 9). A free competitor RNA initially contacts a single free protomer of the Hfq-RNA complex and consecutively replaces the resident RNA in a series of reversible intermediate steps (Vogel & Luisi 2011, Wagner 2013). Rapid dissociation of either RNA is thought to occur from a one-protomer-bound state (Wagner 2013). In this case the rate of binding of the incoming RNA is not limited by the Hfq-RNA dissociation rate but is proportional to the concentration of the free competitor RNAs. At competitor RNA concentrations of 100-200 nanomolar the half-lives of RNA-Hfq complexes are 1-2 min, which allows for fast cycling rates (Fender *et al.*,, 2010; Wagner, 2013; Vogel and

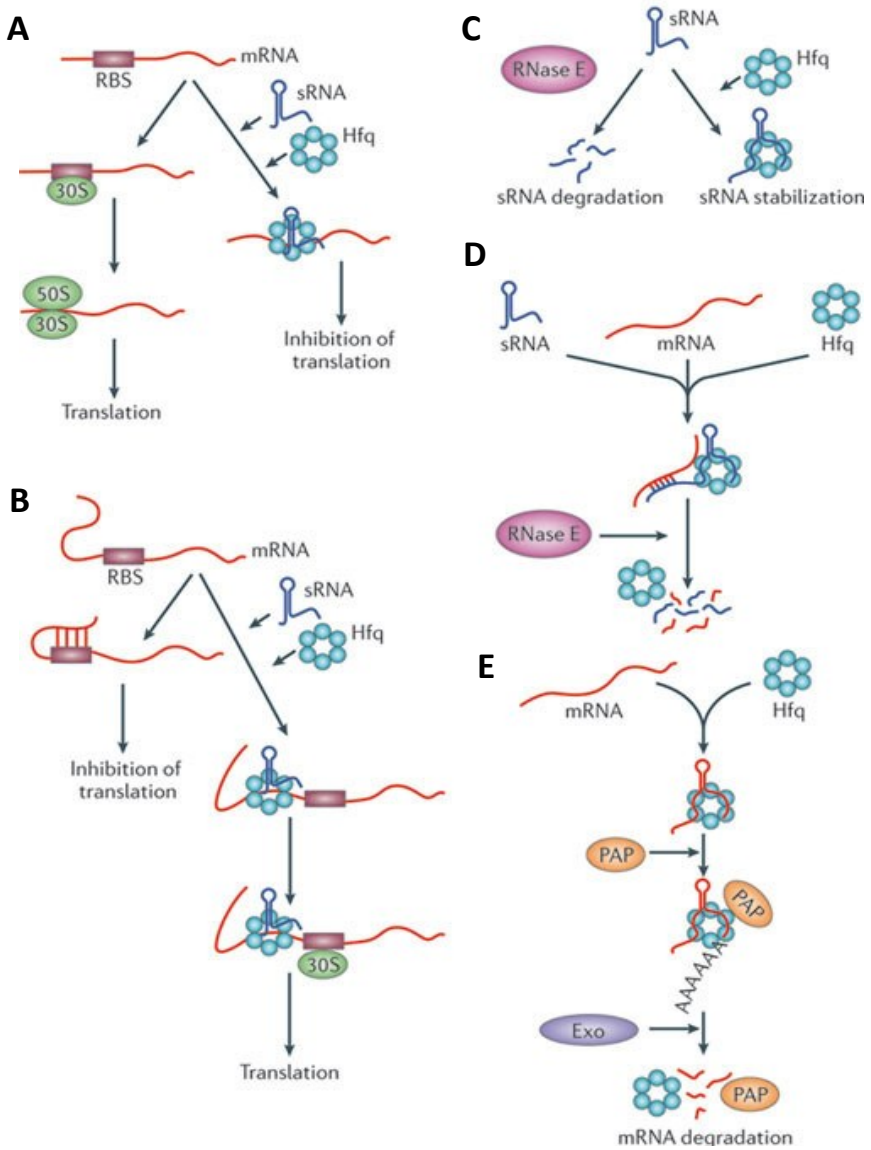

Fig. 10. Modes of action for Hfq-mediated post-transcriptional regulation (Vogel & Luisi 2011). (**A**) Hfq is able to help facilitate binding of a small RNA in proximity to the SD sequence, rendering it inaccessible for ribosome binding. (**B**) Alternatively Hfq can promote mRNA translation by facilitating base-pairing with a small RNA and target mRNA, where base-pairing resolves an inhibitory secondary structure. (**C**) Hfq is known to stabilize and protect RNAs from degradation. (**D**) But Hfq may also promote cleavage and degradation of RNAs. (**E**) In addition Hfq may also stimulate polyadenylation of RNAs, triggering 3'-to-5' exonucleolytic cleavage.

Luisi, 2011). In accordance with its chaperone function, studies have shown that once the RNA duplexes have formed, they remain stable even after Hfq is removed (Altuvia *et al.,* 1998, Kawamoto *et al.,* 2006, Moll *et al.,* 2003b, Zhang *et al.,* 2002).

Binding of Hfq to a target RNA may lead to various modes of action: Hfq may facilitate binding of a sRNA in the proximity of the SD (Shine-Dalgarno) sequence of a target mRNA, thus leaving the SD sequence inaccessible, which leads to translational silencing (Fig. 10A; Altuvia *et al.,* 1998, Mizuno *et al.,* 1984). The opposite may also be the case, where the binding of a sRNA to a target mRNA leads to conformational changes, which exposes the SD sequence and leads to translation. (Soper *et al.,* 2010, Urban & Vogel 2008). (Fig. 10B; Fröhlich & Vogel 2009, Soper *et al.,* 2010). Hfq is able to protect sRNAs from RNase E-mediated degradation (Fig. 10C; Massé *et al.,* 2003, Morita *et al.,* 2005, Pfeiffer *et al.,* 2009), or promote cleavage of some sRNAs in duplex with their target mRNA (Fig. 10D). The latter scenario occurs during repression of iron-regulated genes in *E. coli* by the sRNA RyhB (Večerek *et al.,* 2003). Additionally Hfq may stimulate polyadenylation of some RNAs by poly(A) polymerase (PAP), thereby triggering 3'-to-5' exonucleolytic cleavage by exoribonucleases (Exo) (Fig. 10E; Hankins *et al.,* 2010, Mohanty *et al.,* 2004).

Further Hfq is able to act as a direct repressor of translation in *P. aeruginosa*; during carbon catabolite repression. Hfq is able to bind to mRNAs of catabolic genes and to inhibit their translation by masking the SD sequence (Sonnleitner & Bläsi 2014).

1.7. Regulatory RNAs in Bacteria

Regulatory RNAs in Bacteria present the cell with a tool to regulate and fine-tune gene expression in response to changing environmental conditions. One of the first chromosomally encoded sRNAs with annotated function was MicF, which inhibits translation of the gene, encoding the outer membrane protein OmpF (Mizuno *et al.,* 1984). Regulatory RNAs can generally be divided into 5 major subgroups: riboswitches, thermosensors, CRISPR RNAs, protein-binding and base-pairing RNAs.

Riboswitches are *cis*-acting RNA structural elements in the 5'-untranslated regions (5'-UTR) of mRNAs. They are able to fold into two mutually exclusive conformations and consist of an aptamer region (binding the ligand) and the expression platform (reviewed in Mandal & Breaker 2004, Winkler & Breaker 2003). The binding of ligands to the aptamer region drives changes in the conformation. These structural rearrangements typically involve alternative hairpin structures that form or disrupt transcriptional terminators or anti-terminators, or that occlude or expose ribosome-binding sites (Grundy *et al.,* 2003, Johansen *et al.,* 2003, Rodionov *et al.,* 2003, Sudarsan *et al.,* 2003).

RNA thermometers are *cis*-acting elements which sense changes in temperature and are often located in the 5' UTR of mRNAs. Upon a shift in temperature they alter their conformation, which reveals or masks the RBS of the mRNA, and thus affects translation (Chowdhury *et al.,* 2003, Righetti & Narberhaus 2014). They often control key regulators of virulence and heat- or cold-shock genes (Kortmann & Narberhaus 2012).

CRISPR RNAs (clustered regularly interspaced short palindromic repeats) are a unique class of regulatory RNAs, which confer immunity to phages and mobile genetic elements. They share similarities with the eukaryotic siRNA mediated gene silencing. These systems have been found in around 40% of the Bacteria and 90% of the Archaea (Sorek *et al.,* 2008). The CRISPR array is initially transcribed into a long RNA, which is subsequently processed by the Cascade complex of Cas proteins (Brouns *et al.,* 2008). This forms single stranded crRNAs, which are retained in the Cascade complex (Brouns *et al.,* 2008). Analogous to the eukaryotic RNAi systems, the Cascade complex may then direct base-pairing of the crRNA with the target, leading to cleavage and degradation of the respective nucleic acid (Hale *et al.,* 2009, Marraffini & Sontheimer 2008, 2009).

Protein-binding RNAs act *via* protein sequestration and are present in many Bacteria. Two "classes" of this kind are represented by the CsrB/RsmZ family (Fig. 11A) and by the 6S RNA (Fig. 11B). The 6S RNA was shown to form a stable complex with the RNAP-σ^{70} holoenzyme (Wassarman 2007). During stationary phase growth 6S RNA accumulates and binds the σ^{70} RNA Pol, thereby favoring transcription by the σ^{S} holoenzyme (Wassarman & Storz 2000).

CsrB/RsmY use short sequence motifs to bind to multiple sites of the translational repressors CsrA/RsmA (Valverde *et al.,* 2004), thereby de-repressing their targets. In *P. aeruginosa* this regulatory system governs the switch from acute to a chronic infection (Goodman *et al.,* 2004).

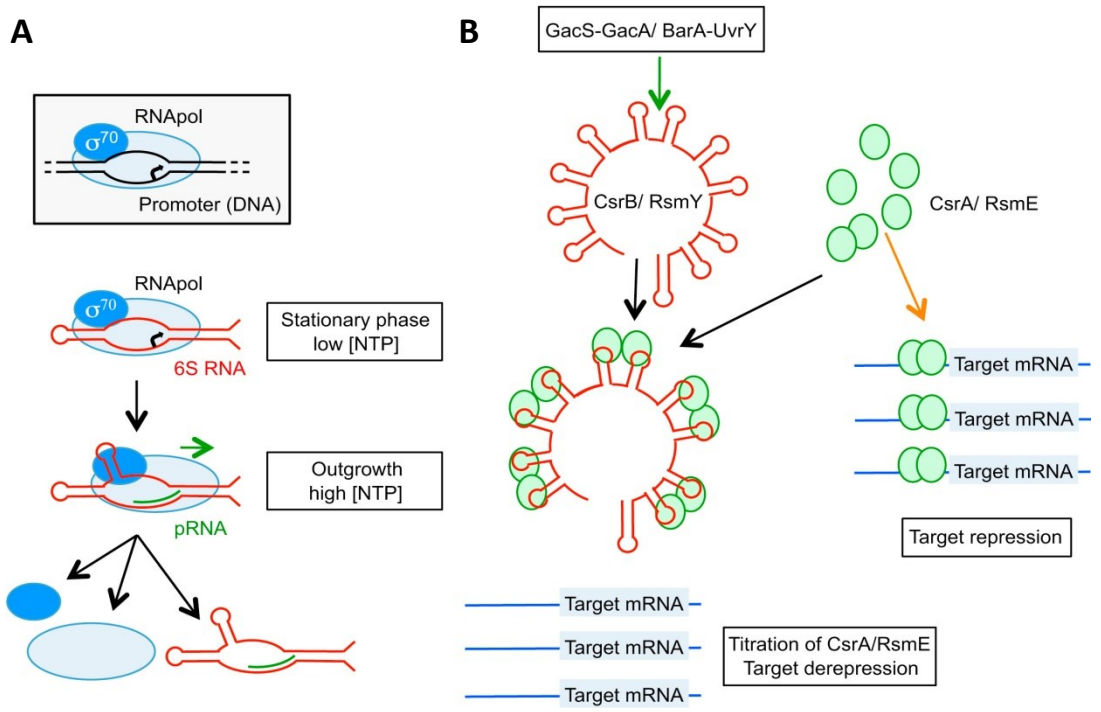

Fig. 11. Modes of action for protein binding RNAs (Wagner & Romby 2015).
(**A**) The 6S RNA mimics σ^{70} open promoters and is able to bind the Eσ^{70} holoenzyme. 6S RNA accumulates during stationary phase in the cells and is then able to sequester most of the Eσ^{70} holoenzyme. This leads to the promotion of transcription from promoters regulated by σ^{S} by altering the competition of Eσ^{70} and Eσ^{S} binding to promoters. (**B**) The CsrB/RsmY RNA uses multiple sequence motifs to bind to several CsrA/RsmE proteins. By binding to CsrA/RsmE it sequesters the translational repressor, thereby de-repressing the target mRNAs.

Another protein binding RNA is CrcZ/CrcY, which regulates carbon catabolite repression (CCR) in *Pseudomonas* (Moreno *et al.,* 2012, Sonnleitner & Bläsi 2014, Sonnleitner *et al.,* 2009). When CCR is alleviated these RNAs accumulate. They use A-rich motifs to bind to and to sequester Hfq, thereby preventing Hfq from blocking the RBS of translationally repressed genes (Sonnleitner & Bläsi 2014).

Base-pairing RNAs can be subdivided into *cis*-encoded and *trans*-encoded base-pairing RNAs. ***Cis*-encoded base-pairing RNAs** (asRNAs) are encoded on the DNA strand opposite of their target RNAs, thereby sharing regions of perfect complementarity with their target (often more than 75nt; Brantl, 2007). They are transcribed as discrete RNA species and function in *trans* as diffusible molecule (Waters & Storz 2009). The proximity of asRNAs to their target seems to be an advantage opposed to sRNAs. This allows transcriptional regulation *via* attenuation *i.e.* the formation of an intrinsic terminator structure (Brantl *et al.,* 1993, Giangrossi *et al.,* 2010) or interference *i.e.* the interaction of two convergent transcription apparatuses (Koroleva *et al.,* 2016). So far transcriptional regulation by asRNAs was only shown to repress targets (Lejars & Hajnsdorf 2020), whereas post-transcriptional regulation was shown to either act positively (Opdyke *et al.,* 2004) or negatively (Kolb *et al.,* 2000).

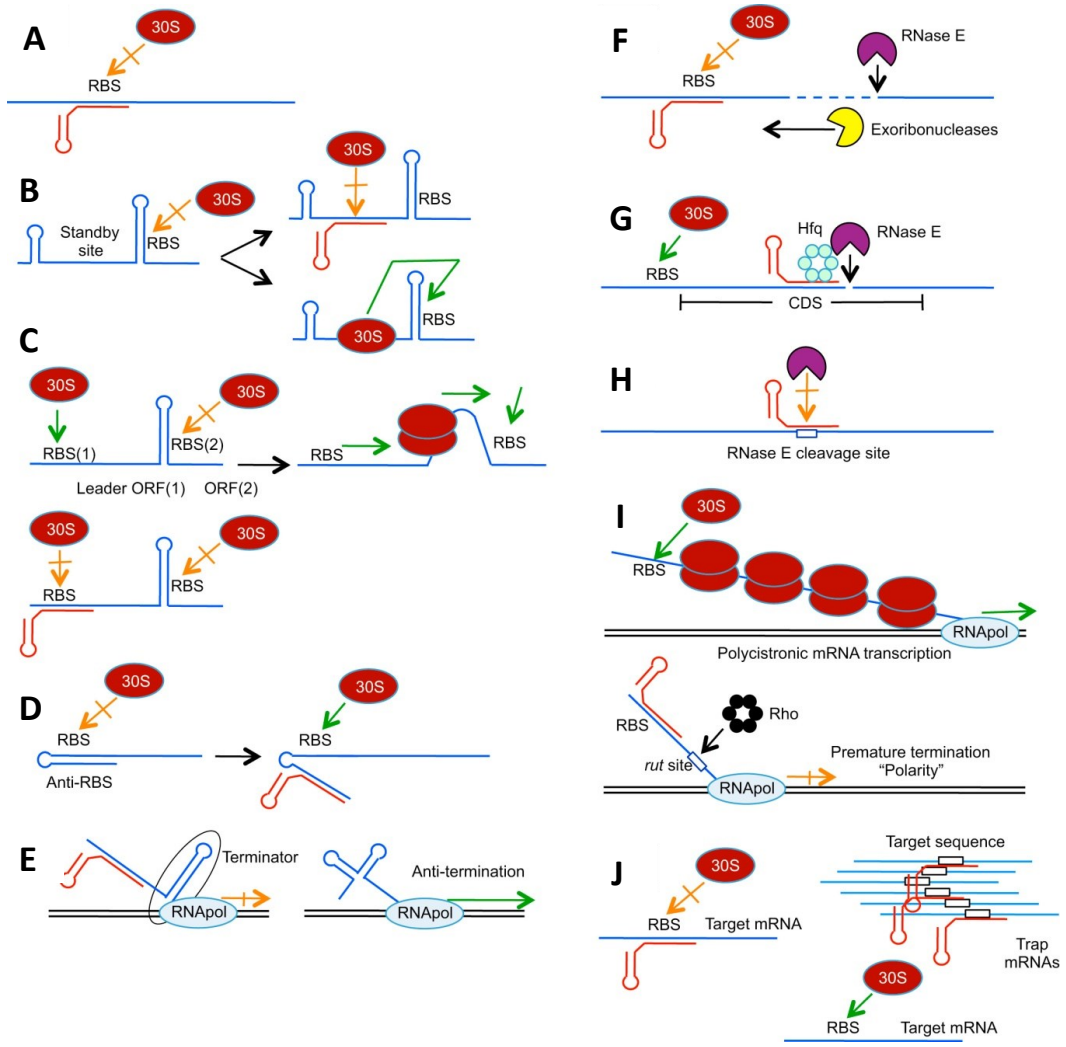

Fig. 12. Mechanisms of action for base-pairing RNAs (Wagner & Romby 2015). (**A**) A regulatory sRNA blocks direct access of the ribosome to the RBS and directly inhibits translation. This is the case for the sRNA MicA (Udekwu *et al.,* 2005). (**B**) The 30S subunit of the ribosome binds to an upstream standby site and translation initiation is achieved by transient breathing, *i.e.* a short transient opening of the stemp loop allows sliding of the standby ribosome onto the RBS. The sRNA binds to the standby site and inhibits translation (*e.g.* the sRNA IstR-1; Darfeuille *et al.,* 2007) (**C**) Indirect inhibition of translation, the RBS (RBS2) is sequestered by a secondary structure. Translation of the ORF is positively coupled to that of an *uof*. The sRNA blocks access of the ribosome to the *uof* thereby inhibiting translation of the ORF (*e.g.* RyhB; Vecerek *et al.,* 2007). (**D**) Direct activation of translation by a sRNA. Here the RBS is sequestered in a secondary structure, binding of the sRNA resolves the secondary structure and translation can occur (e.g. translation of RpoS is activated by the DsrA RNA (Večerek *et al.,* 2009). (**E**) Binding of the small RNA results in premature termination of transcription (*e.g.* RnaG; Giangrossi *et al.,* 2010). (**F**) Translation inhibition is rapidly followed by degradation of the mRNA (*e.g.* SgrS; Vanderpool & Gottesman 2004). (**G**) RNA decay, the small RNA associated with Hfq binds in the CDS of the mRNA. This favors cleavage by RNase E without affecting translation (*e.g.* the sRNA MicC; Bandyra *et al.,* 2012, Pfeiffer *et al.,* 2009). (**H**) Stabilization of RNA, the binding of a sRNA may also protect the RNA from cleavage by RNase E (*e.g.* RydC; Fröhlich *et al.,* 2013). (**I**) Transcriptional polarity: a sRNA binds to the RBS of a polycistronic mRNA and the mRNA becomes devoid of ribosomes. A normally inaccessible Rho-binding site is revealed (*rut*), leading to premature termination (*e.g.* the sRNA ChiX; Figueroa-Bossi *et al.,* 2009). (**J**) sRNA can indirectly activate translation by sequestering an inhibitory RNA acting as a sponge or decoy. This is the case for the sRNA ChiX which is titrated away and degraded by a small intercistronic RNA (Figueroa-Bossi *et al.,* 2009).

Several asRNAs are expressed from bacteriophages, plasmids or transposons, and function to maintain the appropriate copy number of the mobile genetic element (reviewed in Eguchi *et al.,* 1991). This is achieved through a wide range of mechanisms, including inhibition of replication and primer formation as described for plasmid ColE1 RNA I (reviewed in Eguchi *et al.,* 1991, Tomizawa *et al.,* 1981). Another common function is to act as antitoxins and repress the translation of toxins in toxin-antitoxin systems, where the cells which have lost the mobile element are killed (Acuña *et al.,* 2020, Fozo *et al.,* 2008b, 2008a, Kawano *et al.,* 2007).

An important group of asRNAs is involved in the control of catabolism as exemplified by the asRNA dorf, regulating the master regulator of carbon catabolite repression (*crp*) in *E. coli* (Okamoto *et al.,* 1988). The response to acid stress in *E. coli* and degradation of glutamate is under control of the GadX and GadW transcription factors, which are regulated by the asRNA GadY. GadY binds to the discistronic mRNA *gadX-gadW* and triggers cleavage, which leads to stabilization of the *gadX* transcript (Opdyke *et al.,* 2004, Tramonti *et al.,* 2008).

***Trans*-encoded base-pairing RNAs** (sRNAs) only share limited complementarity with their target mRNAs as they are encoded at a different genomic locus as their target. They are able to base-pair with multiple mRNAs and regulate translation or stability of target mRNAs, which allows that a single sRNA is able to mount a global response to *e.g.* changing environmental conditions, similar to a transcription factor but at the post-transcriptional level. Expression typically occurs under very specific conditions; RyhB under conditions of iron limitation (Massé & Gottesman 2002, Massé *et al.,* 2007), OxyS during oxidative stress (Altuvia *et al.,* 1997) or MicA and RybB during outer membrane stress (Douchin *et al.,* 2006, Figueroa-Bossi *et al.,* 2006, Johansen *et al.,* 2006, Papenfort *et al.,* 2006, Udekwu *et al.,* 2005). The base-pairing region typically encompasses 10 - 25 nucleotides, but in all cases only a core of base-pairing nucleotides is needed for regulation (Kawamoto *et al.,* 2006). sRNAs need an RNA chaperone for the binding and regulation to targets (Song *et al.,* 2008). The majority of the regulation is negative, which leads to a repression of target genes. sRNA length ranges from 50 - 300 nucleotides (Waters & Storz 2009) and they can be seen as functionally homologous to the eukaryotic miRNAs. sRNAs encoded in *trans* can be further subdivided by their mechanism of action.

Direct inhibition of translation: The majority of sRNAs use this mechanism for regulation of gene expression. The sRNA base-pairs with the target mRNA and directly inhibits translation initiation, by sequestering the RBS in a secondary structure. This was shown for the Hfq-dependent regulation of OmpA by the small RNA MicA (Fig. 12A; Udekwu, *et al.,*, 2005). Even though the sRNA-mediated repression of translation is the main cause for translational inhibition, the mRNA-sRNA duplex is simultaneously subject to rapid degradation. This is thought to increase the robustness of repression (Morita *et al.,* 2006). For instance, rapid RNAse E-mediated degradation is observed after binding of sRNA SgrS to *ptsG* mRNA (Fig. 12F; Vanderpool, & Gottesman, 2004).

Direct activation of translation: *Trans*-encoded regulatory RNAs may also act by activating translation of the target mRNA. In such cases the RBS is often sequestered by an inhibitory secondary structure in the 5'-UTR. Base-pairing of the sRNA leads to intramolecular remodeling and the RBS is made accessible for the ribosome (Hammer & Bassler 2007, Prévost *et al.,* 2007). For example, the translation of *rpoS* mRNA is activated by the DsrA RNA, which binds in proximity to the RBS and resolves an inhibitory secondary structure (Fig. 12D; Majdalani, *et al.,*, 1998, Večerek, *et al.,*, 2009).

Indirect inhibition of translation: In some 5'-UTR of mRNAs, ribosome standby sites (Fig. 12B) or upstream open reading frames (Fig. 12C) exist (*uof*), which are coupled to translation of the target mRNA. In these cases the sRNA blocks binding of the ribosome to the standby site, or sequesters the RBS of the *uof* leading to translational inhibition of target genes. This is the case for the ferric uptake regulator (Fur) in *E. coli.* The *fur* mRNA is translationally coupled to an *uof*, which is targeted by the sRNA RyhB. Base-pairing of RyhB to the *uof* leads to the inhibition of translation initiation of the *uof* (Fig. 12C), and consequently in translational silencing of *fur* (Vecerek *et al.,* 2007). For the *E. coli tisAB* locus the RBS is not accessible and translation initiation requires ribosome binding to a standby site about 100 nt upstream of the RBS, which is preceded by the sRNA IstR-1 (Fig. 12B; Darfeuille, *et al.,*, 2007). Another mechanism of action is described by the sRNA MicC which binds in the CDS of *Salmonella typhimurium ompD*. This favours cleavage by RNAse E without affecting translation initiation (Fig. 12G; Bandyra, *et al.,*, 2012, Pfeiffer, *et al.,*, 2009).

Indirect activation of translation: Translational coupling may also lead to translational activation, which is employed by the sRNA PhrS (see 1.7.1.6) in *P. aeruginosa* (Sonnleitner *et al.,* 2011). Another mechanism employed is target mimicry, similar to protein binding RNAs. In *Salmonella* the outer membrane porin ChiP is not needed when chitooligosaccaride concentrations are low. Silencing occurs *via* the sRNA ChiX, which binds to the RBS of *chiP* and reveals a usually inaccessible Rho-binding site, leading to Rho-dependent transcription termination (Fig. 12I). If however chitobiose is abundant in the environment, the intergenic region *chbBC* acts as a decoy for ChiX, thereby inhibiting ChiX-mediated repression of ChiP (Fig. 12J; Figueroa-Bossi, *et al.,*, 2009). Finally sRNA binding may stabilize the target mRNA and protect against RNAse E cleavage as shown for RydC (Fig. 12H; Fröhlich, *et al.,*, 2013).

1.7.1 Selection of regulatory RNAs with annotated function in *P. aeruginosa*

1.7.1.1 The small RNA RyhB

RyhB is a 90-nt long sRNA of *E. coli* regulated by Fur. When intracellular iron levels are high, the expression of iron uptake genes is repressed by metallo-Fur (Ernst *et al.,* 1978, Hantke 1981). In contrast when the iron concentration is low, Fur is inactive as Fe^{2+} is required as a cofactor (Hantke & Braun 1998) and repression of iron acquisition genes by Fur is alleviated.

During iron-starvation, RyhB is therefore up-regulated and is able to bind to its target mRNAs through base-pairing, which blocks ribosome binding and thus translation (Geissmann & Touati 2004, Massé & Gottesman 2002, Večerek *et al.,* 2003). In addition it was shown that translational silencing results in rapid turnover of both the target mRNA and the sRNA (Afonyushkin *et al.,* 2005, Moll *et al.,* 2003a, Morita *et al.,* 2006). Further, it was shown that RyhB negatively affects the abundance and stability of *fur* mRNA as well as translation of *fur* by indirect inhibition of translation (Vecerek *et al.,* 2007).

1.7.1.2 The small RNAs PrrF1 and PrrF2

The functional homologues of the *E. coli* sRNA RyhB in *P. aeruginosa* are the PrrF1 and PrrF2 sRNAs (Wilderman *et al.,* 2004). Both sRNAs are preceded by a Fur binding site and are transcribed under iron limiting conditions (Wilderman *et al.,* 2004). Genes regulated by the PrrF sRNAs encode enzymes essential for the defense against iron catalyzed

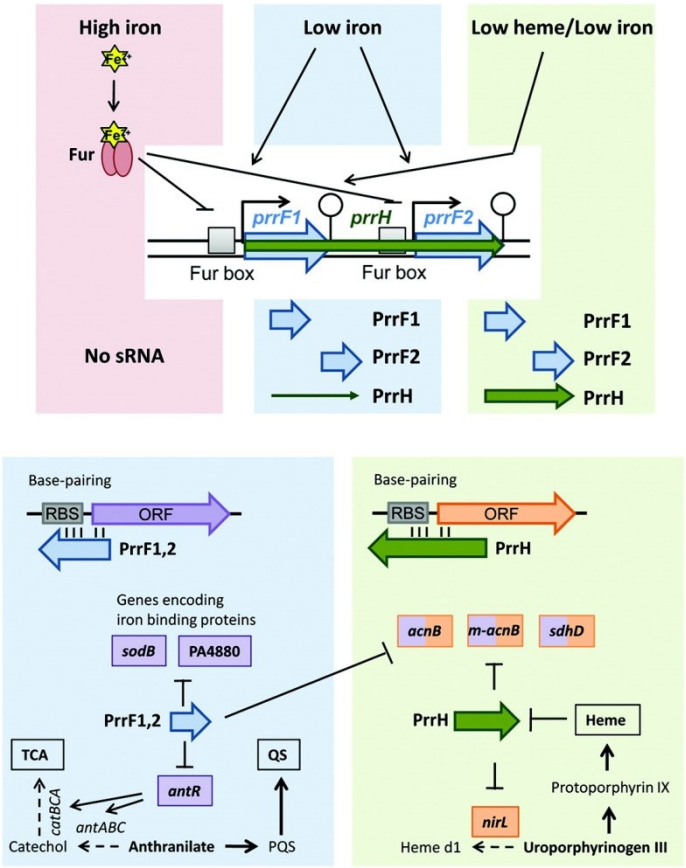

Fig. 13. Model of the regulation of iron and heme by the sRNAs PrrF1-2 and PrrH (Sonnleitner *et al.,* 2012). Under conditions of high iron availability (red box) metallo-Fur blocks transcription from the *prrF1* and *prrF2* promoters. During iron-limitation (blue box) Fur is inactive and transcription of of *prrF1*/*prrF2* can occur. Both RNAs can then repress translation initiation of target mRNAs and iron requiring genes or *antR*, which is the regulator for anthranilate degradation. When iron and heme availability is low (green box) the sRNA PrrH is additionally transcribed from the *prrF1* promoter. PrrH acts similar to PrrF1-2 but additionally represses *nirL*, required from heme d1 biosynthesis. Base-pairing is thought to occur *via* the sequence between the small RNAs.

oxidative stress and genes involved in aerobic and anaerobic respiration and carbon catabolism (Vasil 2007), several of which are involved in the TCA cycle. In addition it was reported that the PrrF sRNAs repress genes involved in anthranilate and catechol degradation (Oglesby *et al.,* 2008). During iron limitation the PrrF sRNAs base-pair with *antR* and *antA* mRNA and prevents their translation. This channels anthranilate into the biosynthetic pathway of the Pseudomonas Quinolone Signal by inhibiting genes required for anthranilate degradation (Djapgne *et al.,* 2018). As a consequence, a PAO1 Δ*prrF1/prrF2* strain shows a decreased production of PQS, which provides a link between iron metabolism, carbon metabolism and quorum sensing in *P. aeruginosa* (Oglesby *et al.,* 2008).

An additional source of iron for *P. aeruginosa* is heme. As with iron, heme uptake and homeostasis are tightly regulated, since a surplus of heme may cause oxidative damage to the cell. The sRNA PrrH is encoded within the *prrF1/prrF2* locus and appears to be induced by heme-regulated anti-termination of *prrF1* transcription (Oglesby-Sherrouse & Vasil 2010). In addition to genes regulated by the PrrF sRNAs, PrrH seems to regulate a specific subset of PrrF independent genes. This specificity is attributed to the intergenic region between *prrF1* and *prrF2* (Sonnleitner *et al.,* 2012).

1.7.1.3 The small RNA ErsA

The 132-nt long small RNA ErsA mediates the response to cell envelope stress and low oxygen environments in *P. aeruginosa* (Ferrara *et al.,* 2015). Transcription was shown to be strongly dependent on the envelope stress responsive alternative sigma factor AlgT/U

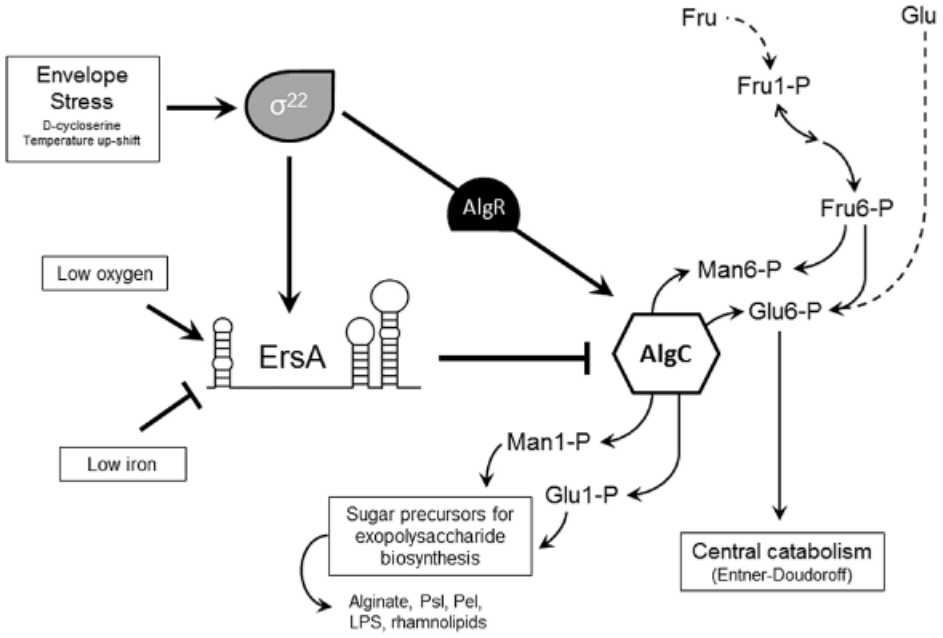

Fig. 14. Model of the modulation of AlgC by the small RNA ErsA (Ferrara *et al.,* 2015). ErsA transcription is induced by either cell-envelope stress in an AlgT/U dependent manner or low oxygen conditions. When ErsA is expressed it is able to base-pair with *algC* mRNA and to inhibit translation in an Hfq-dependent manner.

(Ferrara *et al.,* 2015). AlgT/U is required for the production of exopolysaccaride alginate, which confers the mucoid phenotype giving *P. aeruginosa* an advantage in the cystic fibrosis lung (Potvin *et al.,* 2007). AlgT/U controls the *algD* promoter which drives the expression of the alginate biosynthetic operon (*algD-alg8-alg44-algKEGXLIJFA*; Jain & Ohman 2004). The enzyme AlgC provides the sugar precursors for the biosynthesis of alginate (Fig. 14). ErsA was shown to negatively regulate *algC* expression at the post-transcriptional level in a Hfq-dependent manner. In addition to AlgU/T regulation, *ersA* expression is induced under low-oxygen and it is repressed during iron limitation (Ferrara *et al.,* 2015).

1.7.1.4 The small RNA RgsA

The sRNA RgsA has been identified in two bioinformatic screens (González *et al.,* 2008, Livny *et al.,* 2006), and is transcribed from a RpoS-dependent promoter and indirectly regulated by the GacS/GacA two component system (González *et al.,* 2008). RgsA levels peak during stationary phase (Park *et al.,* 2013). Although RgsA contains a single GGA motif, it is unable to enhance translation of the *hcnA* gene, while it was found that RgsA contributes to the oxidative stress response (González *et al.,* 2008, Park *et al.,* 2013). However, RgsA plays a role in pyocyanin synthesis and swarming motility (Lu *et al.,* 2016). It was demonstrated that RgsA regulates *fis* and *acpP* through direct post-transcriptional regulation in a Hfq-dependant manner (Lu *et al.,* 2016). Recently, it was demonstrated

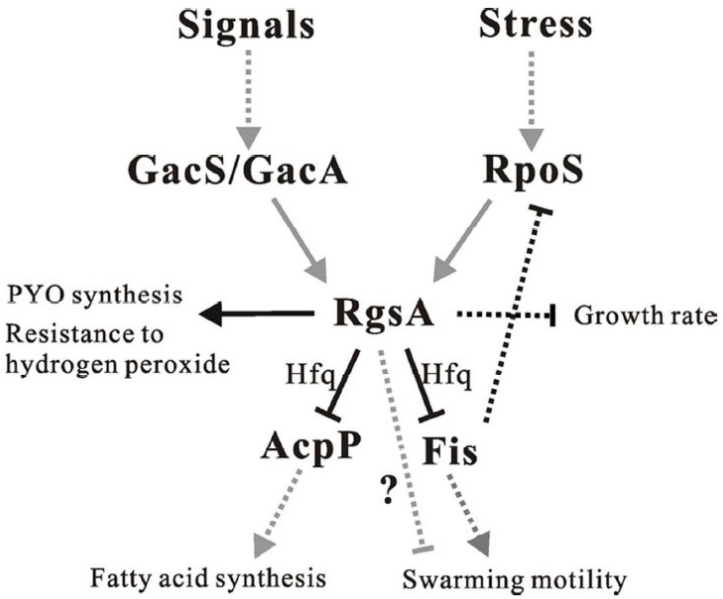

Fig. 15. The small RNA RgsA links fatty acid synthesis and the global transcriptional regulator Fis with the stress sigma factor RpoS (Lu *et al.,* 2016). Two direct regulatory targets of RgsA have been identified, the mRNAs encoding the global transcriptional regulator Fis and the acyl carrier protein AcpP. Synthesis of both Fis and AcpP is down-regulated at the post-transcriptional level by base-pairing. This requires the presence of the RNA chaperone Hfq. This suggests a novel role of the small RNA, where the regulation of Fis is depends on RpoS induction of the *rgsA* gene.

that RgsA negatively regulates *rpoS* expression in an Hfq-dependant manner during the exponential growth phase of *P. aeruginosa* (Lu *et al.,* 2018). However, the physiological role of RgsA remains unclear as no impact on *rpoS* expression during stationary growth was observed (Lu *et al.,* 2018).

1.7.1.5 The small RNA PhrS

PhrS is a 213-nt long sRNA which is expressed during stationary phase growth and oxygen limitation. Transcription is dependent on ANR (see section 1.5) (Sonnleitner *et al.,* 2011). PhrS up-regulates the synthesis of the outer membrane protein OprD and the GroEL chaperone (Sonnleitner *et al.,* 2008). PhrS was shown to induce expression of genes required for pyocyanin and PQS sysnthesis by indirect up-regulation of translation of the *pqsR* gene (Sonnleitner *et al.,* 2011). Translation of *pqsR* was shown to be coupled to an *uof*. Normally the RBS of the *uof* and *pqsR* are inaccessible and sequestered by a stem-loop preventing translation initiation. However, PhrS binding to the *uof* opens the stem-loop and enables translation initiation of the *uof* and thus that of *pqsR* (Fig. 16;

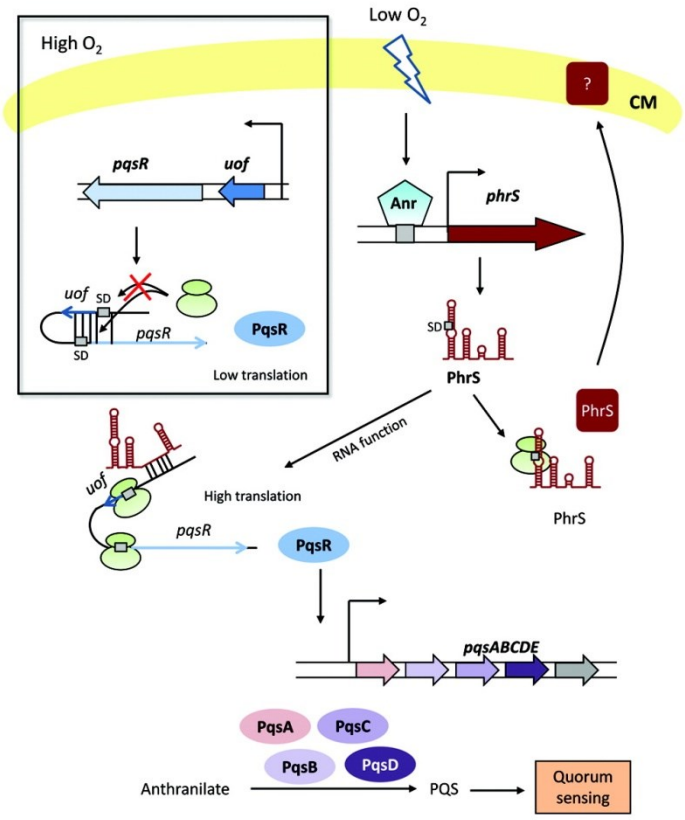

Fig. 16. The small RNA PhrS links oxygen availability with quorum sensing and virulence (Sonnleitner *et al.,* 2011). When oxygen levels are high, translation of the key quorum sensing regulator *pqsR* is impeded by a secondary structure sequestering the RBS. When oxygen levels are low, the transcriptional regulator ANR becomes active and activates transcription of the small RNA PhrS. PhrS is able to base-pair upstream of the *uof*, which is co-transcribed with *pqsR*. This results in indirect activation of *pqsR* translation by PhrS. PqsR then activates transcription of genes involved in biosynthesis of PQS.

Sonnleitner, *et al.*,, 2011). By regulation of *pqsR*, PhrS provides a regulatory link between oxygen levels, quorum sensing and virulence.

In the addition to the function as a sRNA PhrS encodes a conserved 37 amino acid polypeptide (Fig. 16; Sonnleitner, *et al.*,, 2008). Bioinformatic analyses predicted a transmembrane domain and studies revealed the localization in the cytoplasmic membrane (Sonnleitner *et al.,* 2012).

1.7.1.6 The protein sequestering RNAs RsmY and RsmZ

In *P. aeruginosa* a complex regulatory network termed the Gac/Rsm (Fig. 16) system mediates the switch from acute to chronic infection. The translational repressor protein RsmA is able to bind to mRNAs containing GGA motifs. Since this motif often coincides with the RBS, RsmA can inhibit translation initiation (Schubert *et al.,* 2007). RsmA acts as a repressor for many genes involved in chronic infection, including secondary metabolites, virulence factors (*e.g.* type VI secretion, extracellular enzymes), carbon storage compounds and also the regulation of quorum sensing (Brencic & Lory 2009, Burrowes *et al.,* 2006, Kay *et al.,* 2006). On the other hand, many genes required for an acute infection are indirectly up-regulated by RsmA (Brencic & Lory 2009). The availability of free RsmA is regulated by two redundant RNAs, RsmY and RsmZ (Fig. 13). The transcription of RsmY/Z is regulated by the GacA/GacS two component system (Brencic *et al.,* 2009, Goodman *et al.,* 2009).

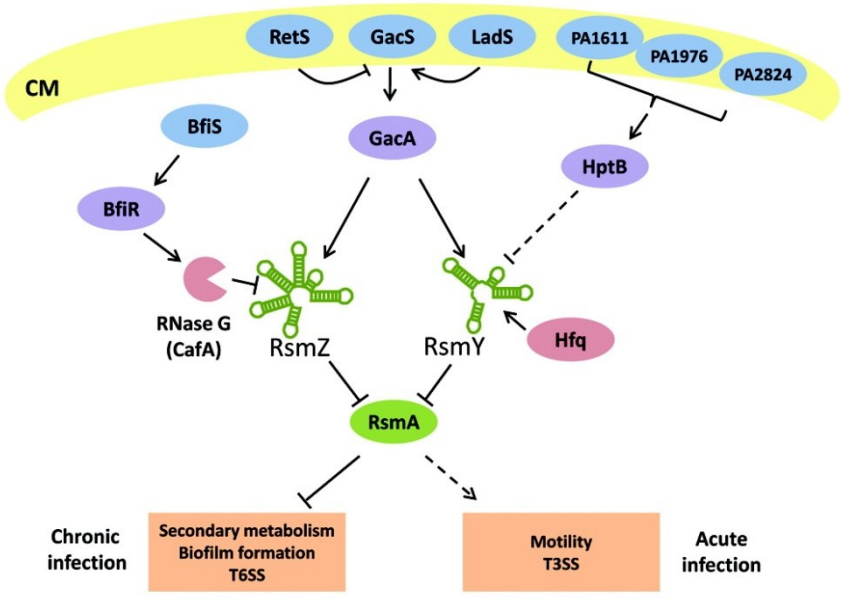

Fig. 17. The protein sequestering RNAs RsmY/Z mediate the switch from acute to chronic infection (Sonnleitner *et al.,* 2012). Both the synthesis and stability of both RNAs is mediated by several different two-component systems in response to a wide variety of environmental signals. GacA mediated activation of transcription occurs on both RNAs, while other factors only impact on one RNA (*e.g.* CafA mediated degradation of RsmZ or Hfq mediated stabilization of RsmY). Both RNAs inhibit the activity of the transcriptional activator RsmA by sequestering the protein, therefore mediating the switch between a chronic and an acute infection.

Although both RNAs share limited sequence similarities, they are structurally conserved where RsmY possesses seven and RsmZ eight GGA motifs, respectively. These are mostly located in loops or between stem-loops (Heeb *et al.,* 2002, Kay *et al.,* 2005, Valverde *et al.,* 2003). Since RsmA binds to these GGA motifs, this allows the RNAs to bind to and to sequester RsmA resulting in the de-repression of genes under control of RsmA (Valverde *et al.,* 2004). While expression of *rsmA* and *rsmY* increases during growth, expression of *rsmZ* is observed mainly during stationary phase (Kay *et al.,* 2005). Both RNAs indirectly influence their own transcription (Lapouge *et al.,* 2008, Ventre *et al.,* 2007), and the stability of RsmY is positively affected by Hfq (Sonnleitner *et al.,* 2006), whereas RsmZ does not interact with Hfq (Sorger-Domenigg *et al.,* 2007). In addition, RsmY/Z are regulated by the sensor kinases RetS and LadS. RetS negatively impacts RsmY/Z transcription by direct interaction with GacS (Goodman *et al.,* 2009). This leads to indirect activation of genes required for acute infection, such as type-III secretion systems or twitching motility (Fig. 16; Laskowski, *et al.,*, 2004, Ventre, *et al.,*, 2006). LadS was shown to counteract RetS leading to an increased transcription of RsmZ, thereby repressing gene products required for an acute infection, and indirectly activating expression of genes required for a chronic infection, such as those required for biofilm formation (Ventre *et al.,* 2006, 2007).

1.7.1.7 The Hfq-sequestering RNA CrcZ

CrcZ is a 407 nt long RNA and involved in regulation of carbon catabolite repression (CCR) in *P. aeruginosa*. CCR ensures the hierarchical utilization of C-sources from "good to poor" carbon sources. It ensures that the gene products required for utilization of non-preferred C-sources are only expressed when no preferred C-source is available. In contrast to *E. coli*, *P. aeruginosa* prefers organic acids such as succinate or amino acids as C-sources (Rojo 2010).

Hfq and the catabolite repression control protein Crc function together as direct translational repressors of genes involved in the metabolism of alternative carbon sources (Sonnleitner & Bläsi 2014, Sonnleitner *et al.,* 2018). Hfq directly interferes with translation initiation of these mRNAs by binding in the proximity of the RBS (Sonnleitner & Bläsi 2014). Crc is able to interact directly with both the mRNA and Hfq enhancing the stability of the Hfq/RNA/Crc complexes (Sonnleitner *et al.,* 2018).

The RNA CrcZ is responsible for alleviation of CCR in *P. aeruginosa*. It is transcribed from a RpoN-dependent promoter and requires the response regulator CbrB of the CbrAB two-component system (Sonnleitner *et al.,* 2009). Transcription and levels of CrcZ are increased in response to the presence of less preferred C-sources as a consequence of activation *via* the CbrAB two-component system. CrcZ encompasses six A-rich binding stretches whereby three were shown to bind to the distal face of Hfq (see section 1.6) with high affinity (Sonnleitner & Bläsi 2014). This allows CrcZ to sequester Hfq, thereby leading to competition of CrcZ with mRNAs for Hfq binding (Sonnleitner & Bläsi 2014).

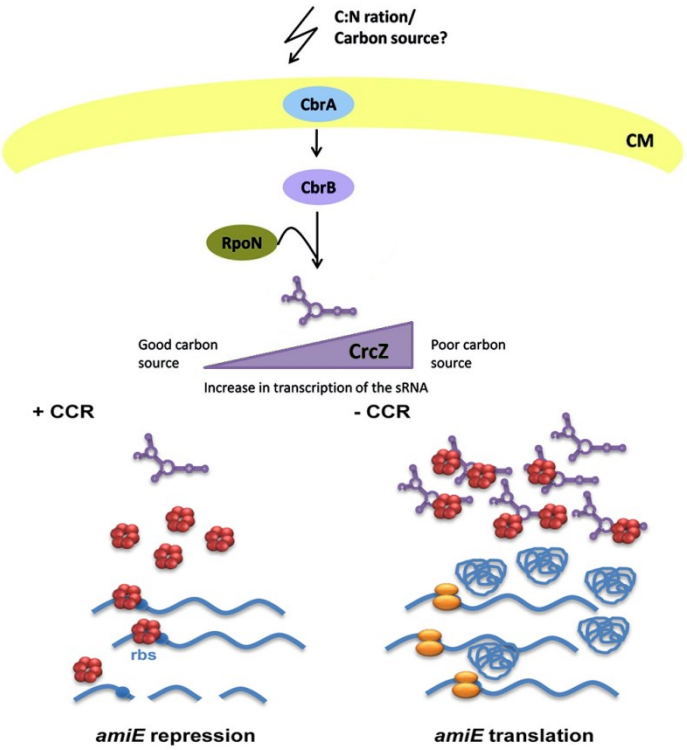

Fig. 18. The regulatory RNA CrcZ regulates carbon catabolite repression in *P. aeruginosa* (modified from Sonnleitner *et al.,* 2011, Sonnleitner & Bläsi 2014). Transcription of CrcZ is activated by the CbrAB two-component system in response to non-preferred carbon sources and is dependent on RpoN. Under conditions where CCR is active (i.e. when the preferred carbon source succinate is present) the levels of CrcZ are low. Hfq can bind in proximity to the RBS of mRNAs encoding catabolic genes and inhibit translation. When however the preferred carbon source is exhausted, transcription of CrcZ is induced. CrcZ binds to Hfq with high affinity and sequesters the protein allowing translation of catabolic genes to occur.

1.8. Aims of the work

The primary aim of this study was to test whether *P. aeruginosa* Hfq mediates riboregulation and whether CrcZ can interfere with this post-transcriptional regulatory mechanism.

As mentioned above, in contrast to *E. coli* Hfq (Hfq_{Ec}), the Hfq protein of *P. aeruginosa* (Hfq_{Pae}) lacks an extended C-terminus. In *E. coli* the Hfq protein facilitates base-pairing between different RNA species and several reports have indicated that Hfq_{Pae} is able to bind to and stabilizes sRNAs and larger protein binding RNAs (Sonnleitner *et al.,* 2002). However, when this study was started, it was not clear whether Hfq_{Pae} can facilitate base-pairing similar to Hfq_{Ec}. Hence, the first question to be addressed was whether Hfq_{Pae} is able to facilitate base-pairing between sRNAs and mRNAs. Previously it was shown that under iron-limiting conditions, the PrrF sRNAs reduce the levels of *antR* mRNA (Oglesby *et al.,* 2008). AntR is an AraC-type regulator activating the transcription of the *antABC* operon encoding genes needed for anthranilate degradation (Fig. 19). Therefore the

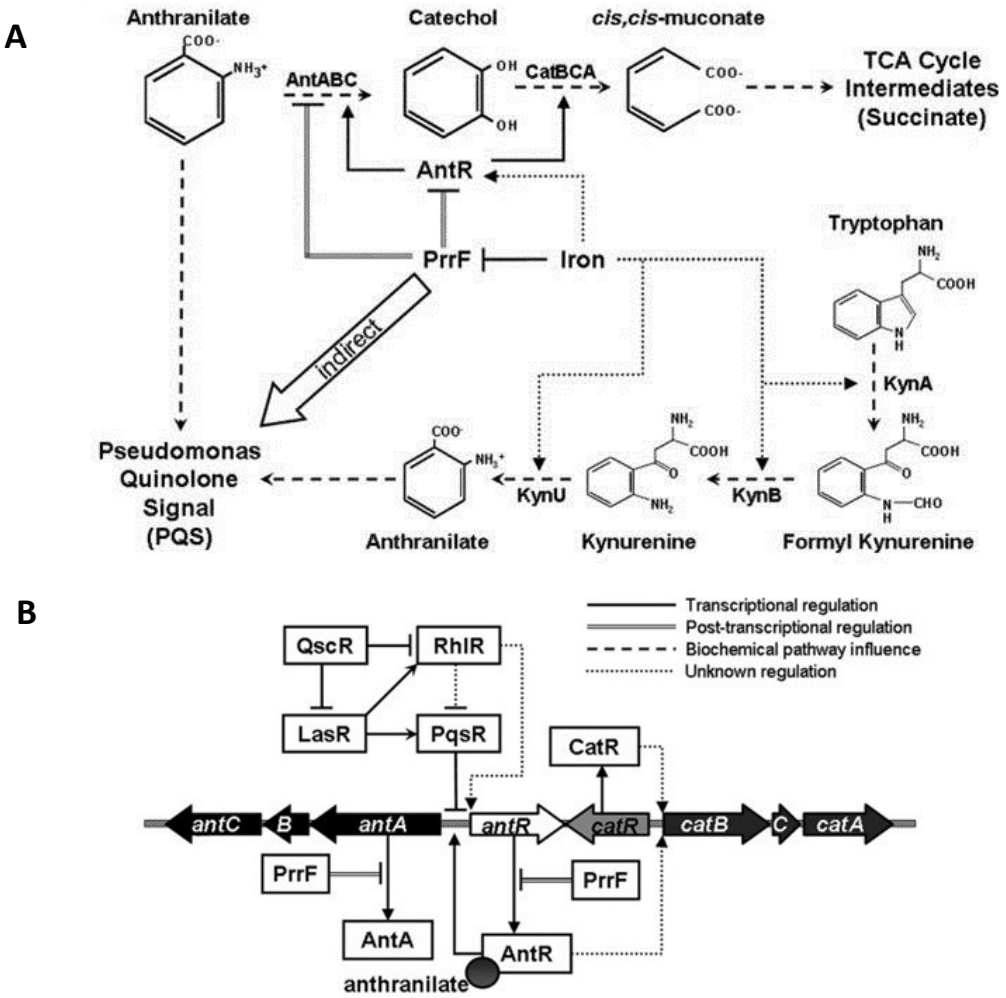

Fig. 19. Iron, PrrF and anthranilate synthesis (Oglesby *et al.,* 2008). (**A**) Under iron-limiting conditions, expression of *antABC* and *catBCA* is repressed saving anthranilate for PQS biosynthesis. Respression of *antA* may occur directly *via* PrrF or inderectly by degradation of *antR* mRNA. (**B**) AntR is an ArcC-type regulator activating transcription of the *antABC* operon and most likely the *catBCA* operon when anthranilate is available. Additional regulation *via* the quorum sensing circuits coordinate expression of genes for anthranilate degradation.

PrrF/*antR* entity was used as a model system to address this question. Furthermore, as CrcZ was shown to sequester Hfq during the relief of carbon catabolite repression, the second question was whether CrcZ is able to interfere with canonical riboregulation.

The second part of this work focused on the regulation of the anaerobic regulator gene *anr*. The transcriptional regulator Anr which regulates anaerobic metabolism Anr (see section 1.5) was shown to regulate at least 36 different genes in *P. aeruginosa* directly (Trunk *et al.,* 2010). In contrast, Hfq was shown to affect the expression of approximately 15% of *P. aeruginosa* genes either directly or indirectly (Sonnleitner *et al.,* 2006). Previous studies of the Bläsi group have revealed that 20 out of 36 genes regulated by Anr are also regulated by Hfq (Sonnleitner *et al.,* 2006). Additionally, both Anr and Hfq show either a positive or a negative regulation of the genes in both regulons, suggesting that some of the genes up-regulated by Hfq are indirectly up-regulated by Anr. It was previously shown that *anr* transcript levels are decreased in an *hfq*$^-$ background (Sonnleitner *et al.,* 2006)

and that expression of *anr* is positively regulated by Hfq (Sonnleitner *et al.,* 2011), however the mechanism remained unclear. In addition, preliminary studies of our group suggested that RpoN is involved in *anr* regulation and that the transcriptional regulator NtrC is essential for transcription of *anr* (K. Kavalchuk, unpublished data). Therefore, the second aim of this study was to verify whether transcription of *anr* is dependent on RpoN and if so, wether RpoN is a target of regulation by Hfq.

2. Materials and Methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1 and 2, respectively.

Table 1. Bacterial strains used in this study.

Strain	Genotype/relevant characteristics	Reference
PAO1	*Wild-type*	(Holloway *et al.,* 1979)
PAO1Δ*hfq*	In frame deletion of the *hfq* coding sequence (5548400- 5548645; Winsor *et al.,* 2011)	(Sonnleitner *et al.,* 2017)
PAO1Δ*crcZ*	Deletion of the *crcZ* coding sequence (5308523-5308682)(Winsor *et al.,* 2011)	(Sonnleitner *et al.,* 2009)
PAO1Δ*prrF1-2*	Deletion of *prrF1* and *prrF2* (5283933-5284353)(Winsor *et al.,* 2011)	(Wilderman *et al.,* 2004)
PAO6358	PAO1*ΔrpoN,* in frame deletion of *rpoN* (4993443-4994343)(Winsor *et al.,* 2011)	(Heurlier *et al.,* 2003)
PAO1Δ*pqsA* CTX-*lux::pqsA*	*pqsA* mutant containing a copy of the *pqsA* promoter linked to the *luxCDABE* genes and inserted into a neutral site in the chromosome	(Fletcher *et al.,* 2007)
E. coli DH5α	*recA1, endA1, hsdR17, deoR, thi-1, supE44, gyrA96, relA1,* Δ(*lacZYA-argF*), *U169*(ϕ80d*lacZ*ΔM15)	(Sambrook & Russell 2001)

Table 2. Plasmids used in this study. Coordinates are given in respect to the transcriptional start site of *rpoN* for pTC*anr2*, pME9851 and pME10353 and in respect to the A(+1) of the start codon for other plasmids.

Plasmid	Genotype/relevant characteristics	Reference
pME6014	pNM481 derivative; pVS1-p15A shuttle vector for translational *lacZ* fusions, Tet^R	(Schnider-Keel & Seematter 2000)
pME6016	pNM481 derivative; pVS1-p15A shuttle vector for transcriptional *lacZ* fusions, Tet^R	(Schnider-Keel & Seematter 2000)
pTL*antR*	Translational fusion of the *antR* gene (nt -311 to +31) in plasmid pME6014, Tet^R	(Sonnleitner *et al.,* 2017)
pME6015P_{tac}	P_{tac} promoter from plasmid pME6032 amplified and cloned into the EcoRI and BamHI site of plasmid pME6015, Tet^R	(Sonnleitner *et al.* 2020)
pP_{tac}*antR*	Translational *antR::lacZ* fusion (nt -56 to +30) in plasmid pME6015P_{tac}, Tet^R	This study
pTC*antR*2	Transcriptional *antR-lacZ* fusion (nt -311 to -53), in plasmid pME6016, Tet^R	(Sonnleitner *et al.,* 2017)
pME4510lacI^qP_{tac}	pME4510 derivative; inducible P_{tac} promoter, lacI^q and lac operator, Gm^R	(Sonnleitner *et al.,* 2017)

pP_{tac}PrrF2	Plasmid for over-expression PrrF2 exactly from the TSS under control of P_{tac}, Gm^R	(Sonnleitner *et al.,* 2017)
pMMB67HE	IncQ expression vector carrying an inducible P_{tac} promoter, Amp^R	(Fürste *et al.,* 1986)
pMMB*crcZ*	pMMB67HE derivative; no RBS for over-expression of CrcZ, Amp^R	(Sonnleitner & Bläsi 2014)
pBBR1*algC::sfGFP*	pBBR1-MCS5 derivative; $P_{LtetO-1}$ --> *algC::sfGFP* Gm^R	(Ferrara *et al.,* 2015)
pTC*anr*2	Transcriptional *anr-lacZ* fusion (-168 to +1) in plasmid pME6016, Tet^R	K. Kavalchuk, unpublished
pME9851	Translational *rpoN::lacZ* fusion, Tet^R	Karine Lapouge Lab
pME10353	Translational *rpoN::lacZ* fusion under control of the P_{tac} promoter, Tet^R	Karine Lapouge Lab

2.2. Culture conditions

The *E. coli* strains were grown in lysogeny broth (Bertani 1951, Luria *et al.,* 1960, Miller 1972) at 37°C unless noted otherwise. Cultures of *P. aeruginosa* were cultivated in BSM minimal medium (7.1.1; Durham & Phibbs 1982) supplemented with 40 mM succinate and 2 µM $FeSO_4$ at 37°C under aerobic conditions unless noted otherwise. For induction of iron limiting conditions instead of 2 µM, 0.2 µM $FeSO_4$ was added to the BSM medium. If required, antibiotics were added to a final concentration of 25 µg/ml tetracycline, 100 µg/ml ampicillin, 15 µg/ml gentamycin for *E. coli* strains and 100 µg/ml tetracycline, 250 µg/ml carbenicillin, 50 µg/ml gentamycin for *P. aeruginosa* strains.

2.3. Construction of plasmids

2.3.1. Construction of pP_{tac}*antR*

Plasmid pP_{tac}*antR* harboring a translational *antR::lacZ* fusion under control of the P_{tac} promoter was constructed as follows. A 107-bp fragment (encompasing nt -56 to +30 in respect to the A(+1) of the start codon) was amplified by PCR using primers W122 (5'-TTT TT**G GAT CC**G AAA ACA GAG CGC CAG CAG-3') and X122 (5'-TTT T**CT GCA G**AC GAT CGG CGA CGG GAT GG-3') and chromosomal DNA of PAO1 as template. The PCR fragment was cleaved with *Bam*HI and *Pst*I, and then ligated into the corresponding sites of plasmid pME6015P_{tac}.

2.3.2. Construction of pP_{tac}PrrF2

Plasmid pTacPrrF2 harboring PrrF2 under control of P_{tac} was constructed as follows. A 130-bp fragment (coordinates 5284206 to 5284319 in the PAO1 genome (Winsor *et al.,* 2011)) was amplified by PCR using primers B127 (5'- **CCC GGG** ACT GGT CGC GAG GCC AG-3') and H120 (5'- TTT TTT T**CT GCA G**GC TAC GTT TTC GCG GGC-3') and chromosomal DNA of PAO1 as template. The PCR fragment was cleaved with *Pst*I and *Sma*I and ligated into the corresponding sites of plasmid pME4510*lacI*^{q}pTac.

2.4. RNA isolation

RNA isolation was performed as described previously (Leoni *et al.,* 1996). After addition of 125 µl RNA protection solution (7.1.2.2) to 1 ml culture, cells were collected by centrifugation (20000 x g, 2 min, 4°C), resuspended in 50 µl of DEPC treated water and lysed by addition of hot-phenol (500 µl phenol with 250 µl lysis buffer (7.1.2.2), prewarmed to 65°C). RNA was extracted using Phenol:Chloroform extraction and precipitated in 2.5 x volumes of 96% ethanol and 0.1 x volumes of 5 M sodium-acetate (pH 5.5). The precipitated nucleic acids were treated for 30 min at 37°C with 40U of RNase-free DNase I, the Phenol:Chloroform extraction was repeated and total RNA was precipitated with 2.5 x volumes of 96% ethanol and 0.1 x volumes of sodium-acetate. The precipitated RNA was resuspended in 20 µl of DEPC-treated water and the concentration was determined using a nanodrop photometer (Nano Drop ND-1000 PEQLAB).

2.5. Northern blot analysis

Unless otherwise noted, 2µg of total RNA were denatured for 10 minutes at 65°C with RNA loading dye (7.1.2.2) and loaded onto the slots of a denaturing 8 M Urea/8% PAA gel. Electrophoresis was performed using 20 mA per gel, the RNA was blotted onto a nylon membrane (Amersham Hybond-N+, GE Healthcare Life Sciences) using the semi-dry transfer method at 12 V for 45 min. RNA was cross-linked twice with UV light (1200 to 0 µJoules). The membrane was pre-hybridized with hybridization buffer for 30 min at 55°C, 3.3 pmol probe were added to the buffer and hybridization was done over night at 55°C. The membrane was washed for 10 minutes with wash buffer I (7.1.2.2) for another 10 min with wash buffer II (7.1.2.2) and finally rinsed with DEPC-treated water. Before exposure to the storage phosphor screen the membrane was air-dried for 30 min. The screen was exposed to the membrane over night, except for the 5S rRNA probe. For 5S rRNA probes the screen was exposed to the membrane for 45 minutes. Visualization of the signals was accomplished using a PhosphorImager (Typhoon FLA 9500, GE Healthcare). For detection of the PrrF sRNA the probe U32_PrrF (5'-GTGATTAGCCTGATGAGGAG–3') was used. For detection of 5S rRNA the probe I26_5SrRNA (5'-CCCCACACTACCATCGGCGATGCGTCG-3') was used, and for detection of CrcZ the probe K3_CrcZ (5'- GCTGGAGTCGTTACGTGTTG-3') was used.

2.6. Oligonucleotide labelling

For labelling of oligonucleotides a total amount of 10 pmol were used. Briefly 10 pmol oligonucleotide labelled with ^{32}P-ATP using the T4 polynucleotide kinase (Thermo). The sample was incubated for 1h at 37°C and subsequently purified using the QIAgen nucleotide removal kit according to the manufacturer's instructions. The labelled oligonucleotide was stored at -20°C.

2.7. β-galactosidase assay

The β-galactosidase was conducted as described previously (Miller 1972) with a few alterations. Briefly cells from 1 ml culture were harvested (20000 x g, 2 min), and the cell pellet was frozen until further use. The cell pellet was resuspended in 1 ml 0.9% NaCl, the OD_{600} was measured using a Hitachi U-1000 spectrophotometer and the cells were permeabilized by addition of 40 µl toluene to 800µl of the sample, 5 min vortexing and incubation of the samples at 37°C for 10 minutes. The samples were diluted in 1:10, 1:50 or 1:100 in Z-buffer (7.1.2.3) and equilibrated for 10 minutes at 37°C. The reaction was started by addition of 200 µl of ONPG solution (7.1.2.3), stopped after sufficient yellow coloration by addition of 500 µl of stop solution and the absorbance at 420nm was measured. The enzymatic activity was determined using the following formula:

$$Miller\ Units = \frac{1000 * OD420}{OD600 * reaction\ time * sample\ volume}$$

2.8. Western blotting

Western blot analysis was performed as described previously (Sonnleitner *et al.,* 2017). Briefly, the cells were collected by centrifugation (20000 x g, 2 min, 24°C) and frozen until further use. Then, the cells were resuspended in 50 µl 1x SDS loading dye per unit of OD_{600} and boiled for 10 minutes at 100°C. 10 µl of each sample were loaded onto a SDS-PAGE gel. Electrophoresis was performed at 20 mA, the proteins were blotted onto a nitrocellulose membrane (GE Healthcare) at 25 V for 30 minutes.

The membrane was blocked for 1 hour at RT in TBS containing 5% milk powder (7.1.2.4). After blocking the membrane was washed 3 x 10 minutes with TBS and incubated with the primary antibody solution (7.1.2.4) for 1 hour at RT. After incubation the washing was repeated and the membrane was incubated for 1 hour with the secondary antibody solution at RT. The membrane was washed for 5 x 10 minutes with TBS and rinsed with alkaline phosphatase buffer (7.1.2.4) afterwards. The membrane was developed by addition of the NBT/BCIP solution until a signal was observed. The ribosomal protein S1 was used as internal control.

2.9. PQS biosensor assay

2.9.1. PQS sample preparation

The PQS sample was prepared as described previously (Fletcher *et al.,* 2007). Briefly, the supernatant (PQS rich medium) of the culture was obtained from 1 ml of sample by centrifugation (20,000 x g, 2 min, 4°C) and passed twice through a 0.2 µm filter. The sample was then frozen until use.

2.9.2. PQS biosensor assay

The PQS biosensor assay was conducted as described previously (Fletcher *et al.,* 2007). Briefly an over-night culture of the biosensor strain was diluted in fresh LB medium to an OD_{600} of 1.0 and further diluted to 0.02. From this standard dilution 100 µl were placed in

the wells of a 96-well plate. To each well either 100 µl 0.9% NaCl (not induced) or 100 µl of PQS rich supernatant were added. Growth (600 nm) and bioluminescence (420 nm) were monitored using a Synergy H1 mircoplate reader. Bioluminescence is shown in RLU (relative light units) per unit of OD_{600}.

2.10. Anaerobic growth of bacterial cultures in a plate-reader

Anaerobic growth of PAO1 cells was performed as follows. Every step until incubation in the plate reader was done under anaerobic conditions in an anaerobic chamber. An over-night culture of the desired strain was washed under anaerobic conditions twice with 0.9% NaCl and diluted to the desired starting OD_{600}. To each well of a 96-well plate 150 µl of a starter culture and Triton-X at a final concentration of 0.001% was added. To sustain anaerobic conditions in the plate reader, 50 µl of sterile paraffin oil was added on top of each well. The growth was monitored using a Synergy H1 microplate reader for 48h.

2.11. AlgC-GFP reporter assay

The *algC*-GFP reporter assay was performed as previously described (Ferrara *et al.,* 2015). Briefly, the Pseudomonas strains were grown in BSM minimum media supplemented with 40 mM succinate (BSM-S) or with 40 mM mannitol (BSM-M) until the desired OD_{600} was reached. 5 ml samples were withdrawn and transferred into test tubes. D-cycloserine was added to a final concentration of 400 µg/ml and the cells were left to grow for additional 2 hours. After 1 and 2 hours a 200 µl sample of the culture was transferred into the wells of a 96-well plate and the fluorescence was measured at 515 nm using a plate reader (1420 Multilabel counter Victor^3 V). The fluorescence units were normalized to the OD_{600} of each sample

3. Results and Discussion

3.1. Hfq_{Pae} stabilizes the two PrrF sRNAs and facilitates canonical riboregulation

In *E. coli* multiple examples of Hfq-mediated riboregulation by sRNAs exist. In *P. aeruginosa* it has been suggested that Hfq_{Pae} may be involved in canonical riboregulation (Ferrara *et al.,* 2015, Lu *et al.,* 2018). Previous studies have shown that the levels of *antR* mRNA are increased in the absence of Hfq (Sonnleitner *et al.,* 2006) and PrrF1-2, respectively (Oglesby *et al.,* 2008). Additionally, the PrrF sRNAs were shown to bind to Hfq_{Pae} (Oglesby *et al.,* 2008). Taken together with the prediction of PrrF binding sites on *antR* mRNA (Oglesby *et al.,* 2008), which overlap with the RBS, we tested if Hfq_{Pae} is capable of supporting canonical riboregulation (*i.e.* base-pairing between a sRNA and its target mRNA in *P. aeruginosa*).

First, a translational *antR::lacZ* reporter gene was constructed, which is encoded by plasmid pTLantR. Using this reporter gene we assayed the expression of β-galactosidase in PAO1 wild-type, PAO1Δ*hfq* and PAO1Δ*prrF1-2* strains, as well as in a *prrF2* over-expressing strain. The strains were cultured in BSM medium supplemented with

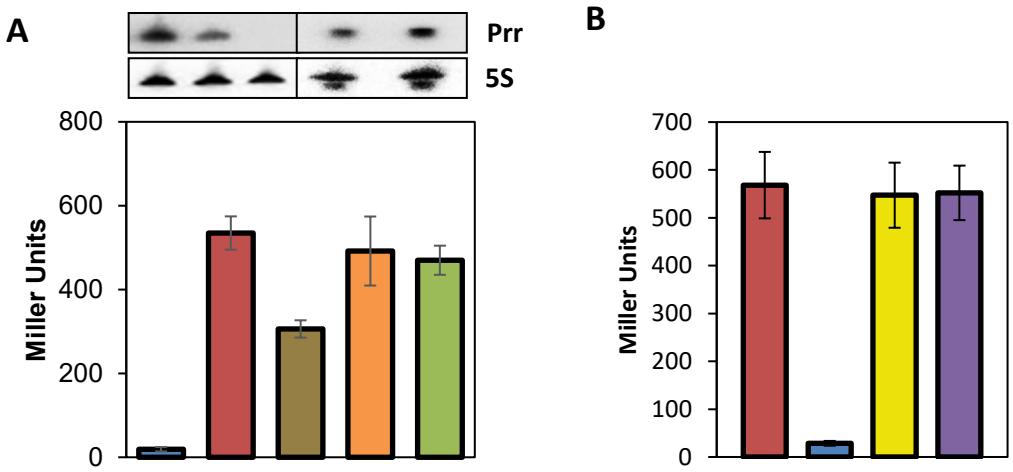

Fig. 20. Hfq_{Pae} participates in canonical riboregulation. (**A**) Repression of *antR* by Hfq and PrrF1-2. The strains were grown in BSM medium supplemented with 40 mM succinate and 0.2 mM anthranilate to an OD_{600} of 2.0. Then, the cells were harvested and the β-glactosidase activity was determined. The bars depict the translation of the *antR*::*lacZ* fusion gene on plasmid pTLantR, in strains PAO1 (blue), PAO1Δ*hfq* (red), PAO1Δ*prrF1-2* (brown), PAO1Δ*hfq* (pME4510) (orange) and PAO1Δ*hfq* (pP_{TAC}PrrF2) (green), respectively. The PrrF2 levels (top) were determined by Northern-blot analysis. 5S rRNA served as a loading control. (**B**) The distal and proximal binding surfaces of Hfq are required for *antR* repression. The translation of the *antR*::*lacZ* fusion gene was determined in strain PAO1Δ*hfq* (pTLantR) strain harboring the control plasmid pME4510 (red), plasmid pME4510hfq_{FLAG} (blue), plasmid pME4510$hfq_{Y25DFLAG}$ (yellow) and pME4510$hfq_{K56AFLAG}$ (purple), respectively. The error bars represent standard deviations of three independent experiments.

40 mM succinate and 0.2 mM anthranilate, which is required for activation of *antR* transcription (Oglesby *et al.,* 2008, Urata *et al.,* 2004). The data presented in Fig. 20A show that repression of *antR* by PrrF requires the presence of Hfq. When both, Hfq and PrrF1-2, are present (Fig. 20A) the translation of *antR* is repressed. However, in the absence of Hfq (Fig. 20A) a 10-fold de-repression of *antR* was observed. Additionally, ectopic over-expression of PrrF in an *hfq* deletion strain (Fig. 20A) did not alter the expression levels of *antR*. Interestingly, an intermediate expression level was observed in a *prrF* deletion strain (Fig. 20A), which was 1.7-fold lower when compared to the PAO1Δ*hfq* strain. Given that Hfq acts as a translational repressor on several mRNAs (Sonnleitner & Bläsi 2014), we speculate that Hfq might represses *antR per se*, however less efficiently as in combination with PrrF1-2. While repression of translation by the small RNA PrrH has been suggested, the *prrF1-2* deletion strain lacks *prrH* (Oglesby-Sherrouse & Vasil 2010).

To further corroborate that Hfq participates in canonical riboregulation, we made use of two variants of Hfq; Hfq_{Y25D} and Hfq_{K56A}. These modifications are known to prevent binding to the distal and proximal RNA binding site on Hfq, respectively (Mikulecky *et al.,* 2004).To this end we complemented a PAO1Δ*hfq* strain with plasmids coding for either Hfq, Hfq_{Y25D} or Hfq_{K56A} and measured the translation of the *antR*::*lacZ* reporter gene using the same experimental conditions as described above. In accordance with our hypothesis neither the Hfq_{K56A} nor the Hfq_{Y25D} mutation was able to restore repression of *antR* (Fig 20B), suggesting that both RNA binding faces are essential for repression of *antR* by Hfq/PrrF. As expected ectopic expression of Hfq_{Pae} was able to restore repression of *antR* (Fig. 20B) while the PAO1Δ*hfq* strain (Fig. 20B) showed the same level of *antR*::*lacZ* translation as the strains complemented with either Hfq_{K56A} or Hfq_{Y25D}.

These results strongly suggest that the Hfq/PrrF entity participates in canonical riboregulation of *antR*. Studies performed in our lab (Sonnleitner *et al.,* 2017) and studies performed by Zheng *et al.* (2016) showed that Hfq_{Pae} stimulates annealing of complementary ribo-oligonucleotides, but to a reduced extent when compared with Hfq_{Ec}. Further evidence is provided by EMSA experiments performed by Sonnleitner *et al.,* 2017, which show that Hfq accelerates duplex formation between PrrF and *antR* mRNA. Taken together this strongly suggested that the PrrF1-2/*antR* entity is a suitable model system to study the interdependence of Hfq-mediated riboregulation and CCR in *P. aeruginosa*.

3.2. CrcZ interferes with riboregulation in *P. aeruginosa*

In *Enterobacteriacae* several examples are known, where regulatory sRNAs are titrated by competing endogenous RNAs. Base-pairing of sRNAs with mRNA (*e.g.* base-pairing and the sequestration of the ChiX sRNA by the *chbBC* mRNA; Figueroa-Bossi *et al.,* 2009), sRNA or even tRNA fragments (*e.g.* the sequestration of RyhB by the $3'ETS^{leuZ}$ RNA in *E. coli*; Lalaouna *et al.,* 2015) other than their primary target may interfere with regulation of the primary target of the corresponding sRNA. An alternative crosstalk is the competition of endogenous RNAs for regulatory RNA-binding proteins. In accordance, hierarchical

binding of mRNAs can exert competition for CsrA, which is exemplified during the control of fimbrial gene expression in *S. typhimurium* (Sterzenbach *et al.,* 2013). In *E. coli* over-expression of several sRNAs was shown to compete with endogenous RNAs for binding to Hfq, which interfered with sRNA mediated post-transcriptional regulation (Moon & Gottesman 2011). Similarly, over-expression of mRNA targets in the absence of their corresponding regulatory RNA, was shown to diminish the regulation of other mRNAs by sRNAs (Hussein *et al.,* 2011).

As mentioned previously, when a less preferred C-source is available, the small RNA CrcZ is synthesized at high levels and binds to and competes for binding with Hfq. The small RNA CrcZ comprises 5 A-rich regions, 3 of which were experimentally verified (Sonnleitner & Bläsi 2014). These provide binding sites for Hfq which is titrated by the small RNA with high affinity. We therefore hypothesized that this competition interferes with Hfq-mediated riboregulation.

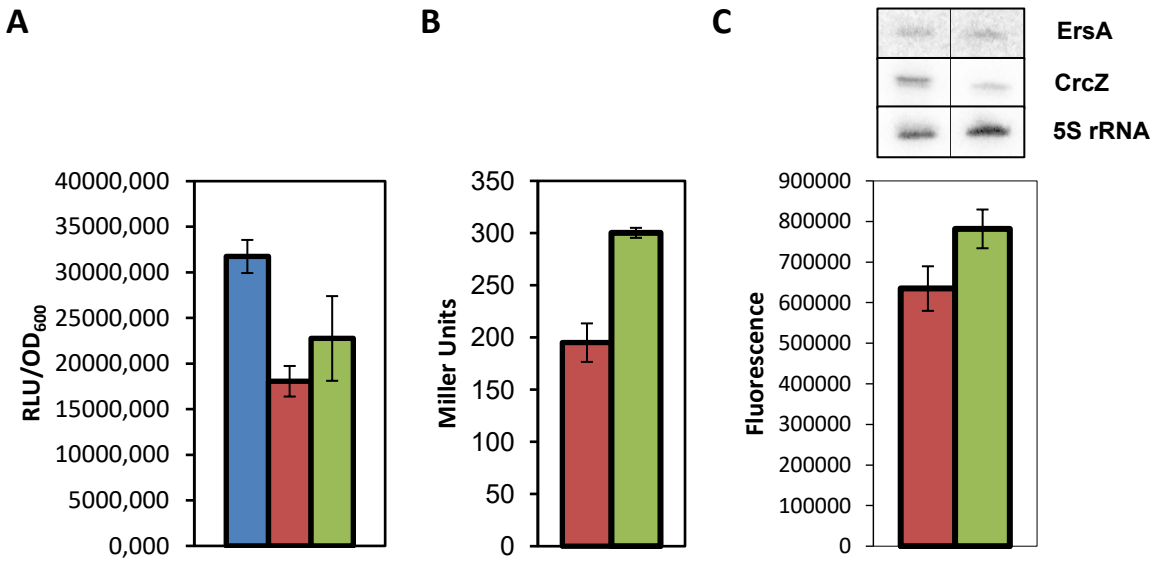

Fig. 21. CrcZ over-expression interferes with Hfq mediated riboregulation in *P. aeruginosa*. (**A**) *antR*-mediated repression of PQS synthesis during *crcZ* over-expression. The strains were grown in BSM medium supplemented with 40 mM succinate and 0.2 mM anthranilate to an OD_{600} of 2.0. The supernatant was then collected and the luciferase activity was determined as luminescence at 420 nm. The bars depict the normalized luciferase activity induced by the PQS rich supernatant of strains PAO1 harboring the control plasmid pMMB67HE (blue), PAO1Δ*hfq* (pMMB67HE) (red) and PAO1 ectopically over-expressing *crcZ* conferred by the plasmid pMMB*crcZ* (green). (**B**) *crcZ* over-expression decreases PrrF/Hfq-mediated *antR* repression when compared to PAO1. The bars depict translation of the *antR*::*lacZ* fusion gene on plasmid pTLantR, in strain PAO1 harboring the control plasmid pMMB67HE (red) and the plasmid pMMB*crcZ* (green), respectively. (**C**) Up-regulation of *algC* during *crcZ* over-expression. The bars depict GFP fluorescence conferred by the translational *algC*::*sfGFP* fusion gene in PAO1 harboring the control plasmid pMMB67HE (red) and the plasmid pMMB*crcZ* (green) respectively. ErsA and CrcZ levels (top) were determined using Northern-blot analysis and the 5S rRNA served as a loading control. The error bars represent standard deviations of three independent experiments.

First, a PQS biosensor assay was employed. Anthranilate serves as an important precursor for PQS (Farrow & Pesci 2007). Additionally, anthranilate can also be utilized as a C-source and can be degraded by the enzymes encoded by the *antABC* and *catBCA* operons (Oglesby *et al.,* 2008). We hypothesized that during repression of *antR* by the Hfq/PrrF entity the degradation of anthranilate is diminished, which would further promote synthesis of PQS. During over-expression of CrcZ, however CrcZ should be able to compete efficiently for Hfq and the repressing effect of PrrF should be alleviated. This would lead to degradation of anthranilate and therefore to lower PQS levels.

To test this, the PQS levels were determined in strains PAO1, PAO1Δ*hfq* and in PAO1 after ectopic over-expression of *crcZ* at an OD_{600} of 2.0 after growth in BSM medium supplemented with 40 mM succinate and 0.2 mM anthranilate. When compared with the wild-typ strain, we indeed observed an up to 1.5-fold decrease in the PQS levels in both, the *hfq* deletion strain and with PAO1 upon over-expression *crcZ* (Fig. 21A).

To further corroborate this result, we measured the expression of an *antR*::*lacZ* reporter fusion in a strain upon ectopic over-expression of *crcZ*. In accordance with the results obtained with the PQS assay, *crcZ* over-expression resulted in 1.5-fold increased translation of the *antR*::*lacZ* reporter gene (Fig. 21B).

To further support our hypothesis that CrcZ interferes with Hfq-mediated riboregulation, we tested if this holds true for another characterized sRNA. To this end we investigated the impact of CrcZ over-expression on the characterized regulation of *algC* by the sRNA

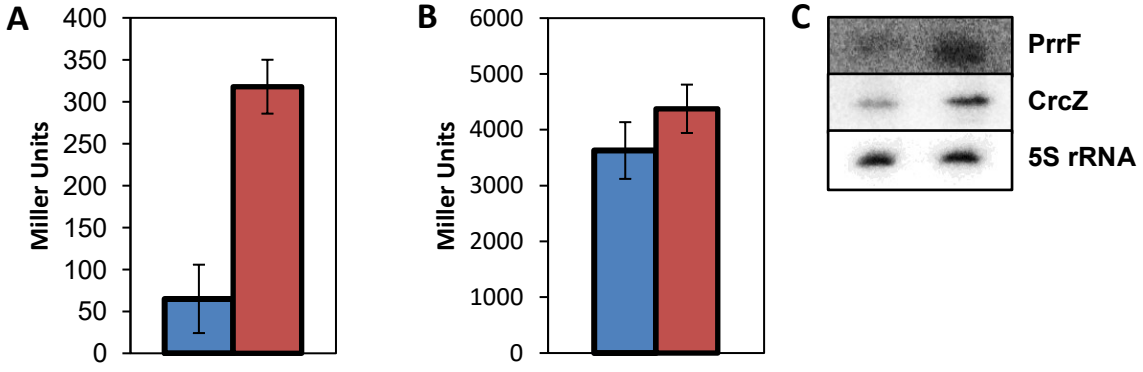

Fig. 22. CrcZ interferes with canonical riboregulation under physiological conditions. (**A**) De-repression of *antR*::*lacZ* translation during growth on a poor C-source. The strains were grown in BSM medium supplemented with 5 mM of either succinate (BSM-S) or mannitol (BSM-M), 5 mM anthranilate and 0.2 µM iron to an OD_{600} of 2.0. Then, the cells were then harvested and the β-glactosidase activity was determined. The bars depict translation of the *antR*::*lacZ* fusion gene in strain PAO1 during growth on BSM-S (blue) and BSM-M (red) respectively. (**B**) The transcription of *antR* remains unchanged during growth in different C-sources. The bars depict the transcriptional efficiency conferred by the transcriptional *antR-lacZ* fusion on plasmid pTC*antR*2 in strain PAO1 during growth on BSM-S (blue) and BSM-M (red) respectively. (**C**) The PrrF and CrcZ levels were determined in strain PAO1 during growth on BSM-S (left) and BSM-M (right) using Northern-blot analysis. 5S rRNA served as a loading control. The error bars represent standard deviations of three independent experiments.

ErsA (Ferrara *et al.,* 2015). Under conditions of cell envelope stress, ErsA represses translation of *algC*. In accordance with our hypothesis, we were able to observe a slight increase in expression of *algC* upon over-expression of *crcZ* (Fig. 21C).

However, the previous results only show that ectopic over-expression of CrcZ interferes with Hfq-mediated riboregulation. Therefore, we tested whether CrcZ is able to interfere with Hfq-mediated riboregulation when the CrcZ levels are closer to physiology. To this end PAO1 wild-type cells were grown in BSM medium supplemented with either a preferred C-source (5 mM succinate) (BSM-S) or a less preferred C-source (5 mM mannitol) (BSM-M) in the presence of 5 mM anthranilate and 0.2 µM iron.

In accordance with the results shown in Fig. 22, *antR*::*lacZ* translation was increased 6-fold when grown in BSM-M when compared to BSM-S (Fig. 22A). In support, expression of a transcriptional *antR-lacZ* reporter was not changed significantly under these conditions (Fig. 22B), suggesting that the increased levels of CrcZ (Fig. 22C) are responsible for the increased expression observed in BSM-M. In agreement, the de-repression of *antR* could be observed even though PrrF levels were increased in BSM-M (Fig. 22C).

3.3. A *crcZ* deletion strain is unable to grow on anthranilate

Next, we asked whether a *crcZ* deletion mutant would be able to utilize anthranilate as a C-source after growth on succinate. During growth on succinate the levels of *crcZ* are low and the Hfq/PrrF entity efficiently represses *antR* translation. Once succinate is depleted the levels of *crcZ* will increase (Fig. 22C) and should interfere with Hfq/PrrF-mediated repression of *antR*. The degradation of anthranilate occurs by enzymes encoded by the *antABC* and *catBCA* operons (Nojiri *et al.,* 2002, Urata *et al.,* 2004) which are activated by AntR (Oglesby *et al.,* 2008). Therefore, we hypothesized that a *crcZ* deletion strain would be unable to utilize anthranilate as a C-source after succinate was depleted. To test this, we monitored growth of the PAO1 wild-type, the PAO1Δ*hfq* and the PAO1Δ*crcZ* deletion strain in BSM-S supplemented with 5 mM anthranilate. Additionally, the expression of the *antR*::*lacZ* reporter gene was monitored before and after depletion of succinate.

In accordance with our hypothesis, we observed an abolishment of growth of the PAO1Δ*crcZ* strain after depletion of succinate (Fig. 23A, triangles), while the PAO1 strain was able to utilize anthranilate (Fig. 23A, boxes). The PAO1Δ*hfq* strain did not show a lag phase after depletion of succinate. This is in accordance with the role of Hfq in CCR (Sonnleitner & Bläsi 2014). In agreement with the abolishment of growth, the translation of *antR* in the PAO1 strain was 3-fold increased after depletion of succinate during growth on anthranilate (Fig. 23B). We attribute this phenotype to the increased levels of CrcZ after depletion of succinate and to the resulting competition of CrcZ for Hfq (Fig. 23C). However, it was reported that under conditions of growth arrest (Yeom *et al.,* 2014) or during stationary phase (Choi *et al.,* 2011) transcription of *antR* is up-regulated. This prompted us to construct a translational *antR*::*lacZ* fusion gene under the control of the P_{tac} promoter to avoid regulation at the transcriptional level. As expected, we observed

an increase in *antR* translation in the wild-type strain after depletion of succinate. Moreover, the PAO1Δ*crcZ* strain showed an even stronger repression of *antR* translation during growth on succinate, when compared to the wild type. However, when succinate was depleted we could observe an increase in expression of *antR* to wild-type levels (Fig. 24). We hypothesized, that the large transcript quantities produced by the P_{tac} promoter could saturate the system, leading to less efficient repression of *antR* translation by the Hfq/PrrF entity. Additional experiments were performed using qRT-PCR to detect the *antR* transcript levels in strains without the reporter plasmids. This showed, that the *antR* levels were severely reduced in the PAO1Δ*crcZ* strain suggesting efficient repression of *antR* translation by the Hfq/PrrF entity (data not shown).

This study extends the role of CrcZ in *P. aeruginosa* in that CrcZ interferes with riboregulation, *e.g.* with repression of *antR* translation by the Hfq/PrrF1-2 entity. Consequently, AntR is synthesized and can induce transcription of the *antABC* and *catBCA* operons (Oglesby *et al.,* 2008) required for anthranilate degradation. Moreover, this study indicates that CrcZ is capable of efficiently competing with other RNAs for Hfq-binding,

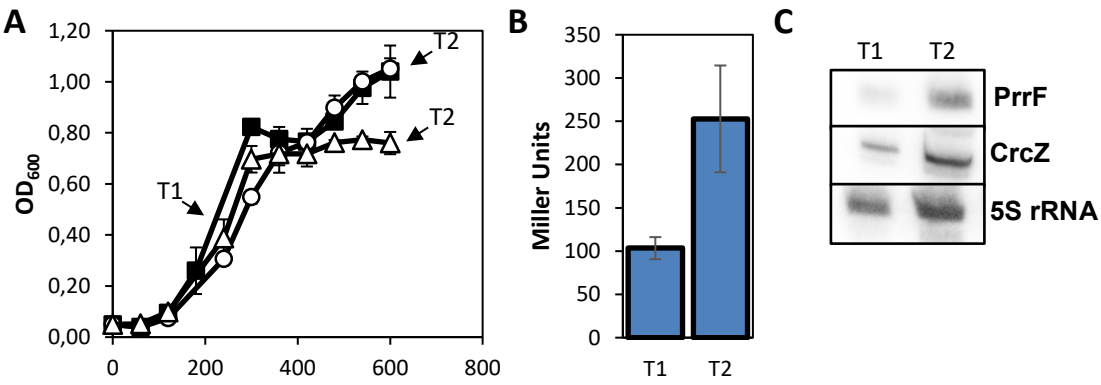

Fig. 23. Diauxic growth with anthranilate is abolished in the *crcZ* deletion strain. (**A**) The utilization of anthranilate as C-source is abolished in the PAO1*ΔcrcZ* strain. Diauxic growth is observed in the wild-type strain, while the *Δhfq* strain shows no lag phase after depletion of succinate. However, the *ΔcrcZ* strain is unable to continue growth on anthranilate after depletion of succinate. The strains were grown in BSM medium supplemented with 5 mM succinate and 5 mM anthranilate. Depicted is the cell density measured at 600 nm of strains PAO1 (■), PAO1*Δhfq* (○) and PAO1*ΔcrcZ* (△), respectively. (**B**) Up-regulation of *antR* after depletion of succinate. The cells were harvested at an OD_{600} of 0.5 (T1) and at an OD_{600} of 1.0 (T2), respectively and the β-galactosidase activity was determined. The bars depict the translation of the *antR*::*lacZ* fusion harbored on plasmid pTLantR in strain PAO1 during diauxic growth on succinate (T1) and on anthranilate (T2), respectively. (**C**) The PrrF and CrcZ levels were determined in strain PAO1 before depletion of succinate (left) and after depletion of succinate (right) using Northern-blot analysis. The 5S rRNA served as a loading control. The error bars represent standard deviations of three independent experiments.

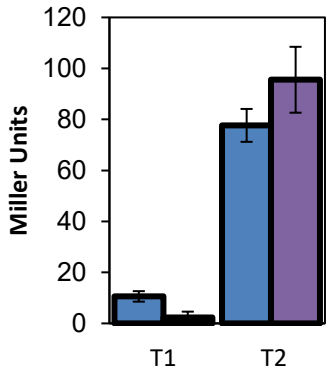

Fig. 24. Translation levels of the *antR*::*lacZ* fusion gene increase to wild type levels after depletion of succinate when under control of P_{tac}. The strains were grown in BSM medium supplemented with 5 mM succinate and 5 mM anthranilate. The PAO1 cells were harvested at an OD_{600} of 0.5 (T1) and 1.0 (T2), respectively. The PAO1Δ*crcZ* cells were harvested at an OD_{600} of 0.5 (T1) and after incubation for 10 hours (T2), respectively. Then the β-galactosidase activity was determined with both strains. The bars depict the translation of the *antR*::*lacZ* fusion under the control of P_{tac} harbored on plasmid pP_{tac}antR in strains PAO1 (blue) and PAO1Δ*crcZ*

and therefore poses the question which other Hfq-mediated pathways are cross-regulated by CrcZ. Subsequent studies have shown, that *P. aeruginosa* strains PAO1 and PA14 show greater susceptibility to antibiotics after addition of a poor C-source (Pusic *et al.,* 2018, Sonnleitner *et al.,* 2020). Thus, the regulation of CrcZ could be a viable target to increase the susceptibility of *P. aeruginosa* to antibiotics. In addition it was shown, that the absence of Hfq results in less efficient formation of aerobic (Pusic *et al.,* 2018) and anoxic biofilms (Pusic *et al.,* 2016).

3.4. Growth of a PAO1Δ*rpoN* deletion mutant is severely attenuated during anaerobic growth

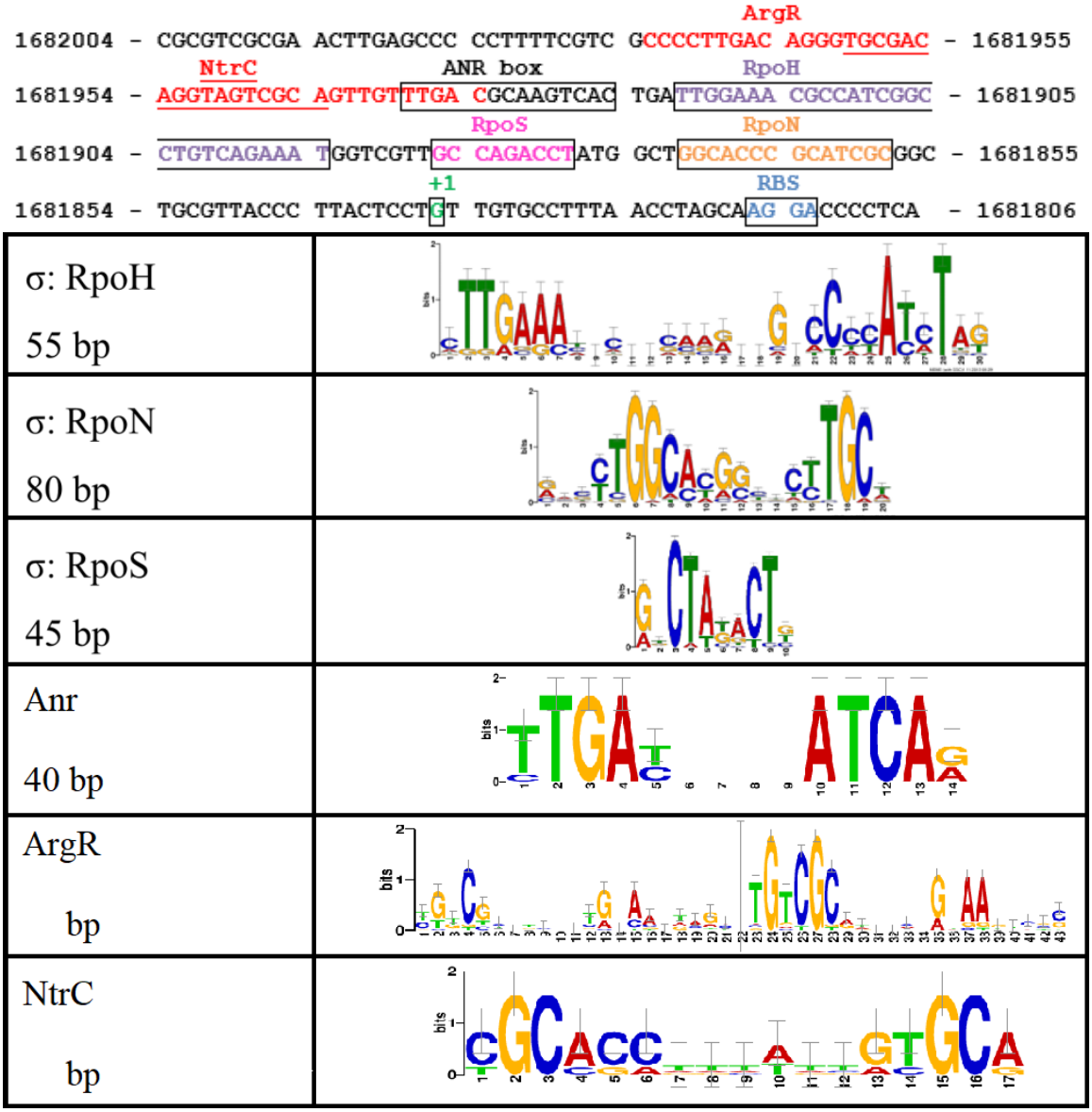

Fig. 25. Top: Promoter sequence of *anr* with binding sites for possible regulators. Several possible binding sites for regulators could be identified in the *anr* promoter. Marked in red is a possible binding site for the AraC-like regulator ArgR responsible for arginine fermentation. In addition, a NtrC binding motif (red, underlined), the response regulator of the NtrBC two-component system. Overlapping with the ArgR binding motif is a putative ANR-box (boxed) indicating possible autoregulation of *anr*. 21-bp upstream of the transcriptional start site (green) is a possible RpoN-box (yellow). Additionally, a potential RpoS binding site (pink) and a possible RpoH binding site (purple) are predicted. Genome coordinates are taken from Winsor *et al.,* 2011.
Bottom (modified from Schulz *et al.,* 2015): Consensus binding motifs for RpoH, RpoN and RpoS taken from Schulz *et al.* (2015). The consensus sequences for Anr, ArgR and NtrC were visualized using WebLogo (Crooks *et al.,* 2004). The data used for visualization were taken from the following studies; Anr (Winteler & Haas 1996); ArgR (Lu *et al.,* 2004, Park *et al.,* 1997); NtrC (Ferro-Luzzi Ames & Nikaido 1985, Hervás *et al.,* 2009).

We were able to identify several potential binding motifs for the following transcriptional regulators in the promoter sequence of *anr* (Fig. 25, top; Winsor, *et al.,* 2011): ArgR, NtrC, RpoN and a possible ANR binding motif, possibly indicating autoregulation. Preliminary studies of our laboratory revealed no differential transcription of *anr* in an *argR* mutant, as well as no autoregulation by Anr (K. Kavalchuk, unpublished). Although the putative recognition site for RpoN was previously identified in the upstream region of the *anr* gene, it remained uncertain whether transcription of *anr* is RpoN dependent (Savioz *et al.,* 1993). However, more recently Schulz *et al.,* (2015) gained evidence for an interaction between RpoN and the *anr* promoter in strain PA14 using CHIP-seq analysis.

To gain first insights, if *anr* transcription is regulated by RpoN we decided to test whether the growth of a *rpoN* mutant strain is affected during anaerobic growth.

As shown in Fig. 26A, during growth under aerobic conditions the strain PAO6358 (PAO1Δ*rpoN*) did not show an apparent growth phenotype, however it was severely inhibited in growth under anaerobic conditions (Fig. 26B). Taken together with previous data from our laboratory that transcription of a*nr* is dependent of the NtrC response regulator (K. Kavalchuk, unpublished), our data strongly suggested that transcription of *anr* requires RpoN.

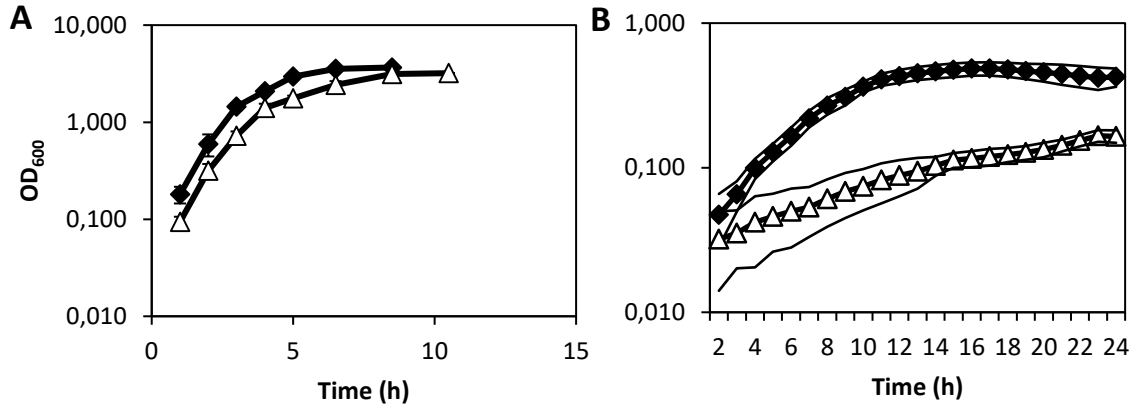

Fig. 26. Growth of strain PAO6358 is severely attenuated under anaerobic conditions. (**A**) Aerobic growth of strain PAO6358 (PAOΔ1*rpoN*) is not impaired when compared to the wild-type starin. The strains were grown in LB medium supplemented with 0.2% of glutamine. The graphs depict the cell density measured at 600 nm of strains PAO1 (◆) and PAO6358 (△), respectively. (**B**) Growth of PAO6358 under anaerobic conditions is severely attenuated when compared to PAO1. The strains were grown in a 96-well plate (see 2.10) in LB medium supplemented with 0.2% of glutamine and 100 mM KNO_3. The graphs depict cell density measured at 600 nm of strains PAO1 (◆) and PAO6358 (△), respectively.The error bars represent standard deviations of three (**A**) and eight (**B**) independent experiments, respectively.

3.5. Transcription of *anr* is dependent on RpoN

To further verify whether the inhibition of anaerobic growth of the *rpoN* deletion mutant was due to a decreased transcription of *anr*, we decided to monitor the levels of *anr* transcription using a transcriptional *anr-lacZ* reporter gene encoded by plasmid pTC*anr*2. The strains were cultured in LB medium supplemented with 0.2% glutamine and 100 mM KNO_3. The cells were harvested before the formation of visible biofilms, 5 and 10 hours after inoculation at an OD_{600} of 0.1. A severe down-regulation of *anr* transcription was observed under these conditions (Fig. 27A). Preliminary experiments addressing the possible regulation of *rpoN* translation by Hfq from the Lapouge laboratory (University of Lausanne) were carried out in BSM minimal medium (K. Lapouge, unpublished). Therefore, we tested whether the down-regulation of *anr* transcription in the Δ*rpoN* strain also occurs under these conditions. The cells were grown in BSM medium supplemented with 0.2% glutamine and 100 mM KNO_3 and harvested 10 and 22 hours after the inoculation to an OD_{600} of 0.1. As expected, the transcription of *anr* was also decreased in minimal medium up to 2-fold in the *rpoN* deletion strain (Fig. 27B). The experiments conducted by Sonnleitner *et al.,* (2011) showed that *anr* is positively

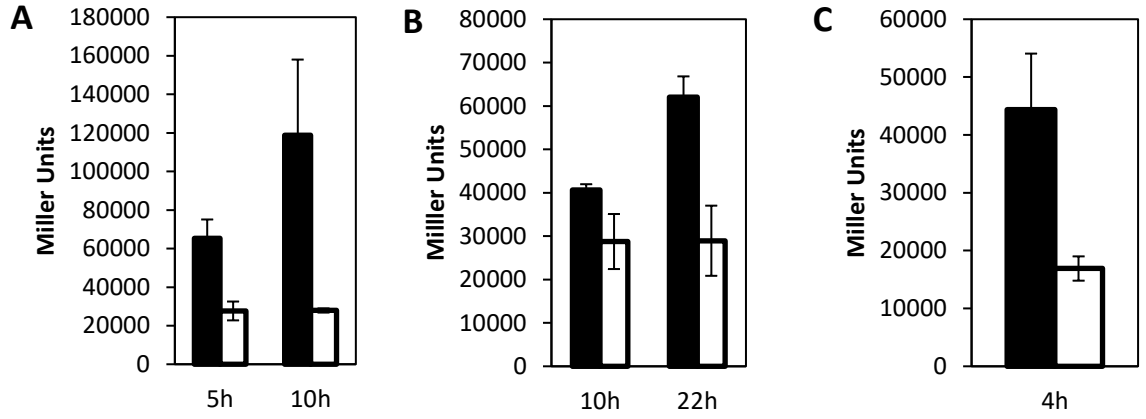

Fig. 27. Efficient transcription of a*nr* is dependent on RpoN. (**A**) Transcription of the *anr-lacZ* reporter gene is decreased in strain PAO6358 up to 4-fold in anaerobic conditions and full medium when compared with PAO1. The strains were grown anaerobically in LB medium supplemented with 0.2% glutamine and 100 mM KNO_3. The cells were harvested and the β-galactosidase activity was determined 5 and 10 hours after inoculation at an OD_{600} of 0.1. (**B**) Transcription of *anr* in strain PAO6358 is decreased up to 2-fold during anaerobic growth in minimal medium when compared with PAO1. The strains were grown anaerobically in BSM medium supplemented with 0.2% glutamine and 100 mM KNO_3. The cells were harvested and the β-galactosidase activity was determined 10 and 22 hours after inoculation at an OD_{600} of 0.1. (**C**) Transcription of *anr* is 3-fold decreased in strain PAO6358 when compared with PAO1 when grown aerobically in full medium. The strains were grown aerobically in LB medium supplemented with 0.2% glutamine. The cells were harvested and the β-galactosidase activity was determined 4 hours after reaching an OD_{600} of 2.5. The bars depict the β-galactosidase activity conferred by the transcriptional *antR-lacZ* fusion gene in strains PAO1 (black bars) and PAO6358 (PAO1Δ*rpoN*; white bars), respectively. The error bars represent standard deviations of three independent experiments.

regulated by Hfq in LB medium under aerobic conditions. Therefore, we tested whether the decreased *anr* transcription observed with the Δ*rpoN* strain does also occur under these conditions. The transcription levels of *anr* were determined 2 and 4 hours after the cells had reached an OD_{600} of 2.5. This is in accordance with a study, showing that oxygen limitation in culture flasks occurs late in stationary phase (Schiefelbein *et al.,* 2013). Interestingly, a down-regulation of *anr* transcription could also be observed under aerobic conditions in the late stationary phase (Fig. 27C). It should be noted, that while *anr* transcription in the *rpoN* deletion strain was not abolished, the levels of transcription normalized to the cell density were constant. This is in accordance with a previous study in which RpoN was shown to be dispensable for denitrification in *P. aeruginosa* (Heurlier *et al.,* 2003). Therefore, we conclude that transcription of *anr* in *P. aeruginosa* is driven by RpoN, however it does not seem to be essential.

Interestingly, the promoter sequence of *anr* additionally revealed a -35 5'-TTGCCA-3' sequence showing sequence similarity to the putative RpoD recognition motif (5'-TTGACC-3'). A corresponding -10 5'-TATAAT-3' sequence (Domínguez-Cuevas & Marqués 2004) is however missing, which might suggest that RpoD is not responsible for the observed transcription of *anr* in the PAO1Δ*rpoN* strain (Fig. 27).

In addition to a RpoN recognition motif, putative recognition sites for RpoH and RpoS were identified in the promoter region of *anr* (Fig. 25, top). Previous experiments in our group have revealed that RpoS does not significantly impact *anr* transcription (K. Kavalchuk, unpublished data). In *E. coli,* RpoH is responsible for the the function of the heat shock induction response (Arsène *et al.,* 2000), however not much is known about RpoH in *P. aeruginosa*. It was shown that PAO1 *rpoH* is able to complement an *E. coli rpoH* mutant strain for growth of phage λ at 30°C (Benvenisti *et al.,* 1995), and that induction of *rpoH* occurred during heat shock in *P. putida* similar to *E. coli* (Aramaki *et al.,* 2001). Therefore, experiments are currently underway to test whether the observed background transcription of *anr* is mediated by RpoH.

3.6. Hfq does not significantly impact RpoN expression

Previous studies have revealed that the expression of *anr* is down-regulated in a *hfq*$^-$ background (Sonnleitner *et al.,* 2011). Additionally, microarray data (Sonnleitner *et al.,* 2006) revealed many genes differentially expressed in a *hfq* mutant that are up- or down-regulated in the same way as in an *anr* mutant. Taken together with our results, that transcription of *anr* is at least partially mediated by RpoN we hypothesized that Hfq regulates *anr* expression *via* RpoN. In addition, we have shown that the up-regulation of *anr* transcription by RpoN also occurs in late stationary phase of cells grown under aerobic conditions.

We first determined the β-galactosidase activity conferred by a translational *rpoN*::*lacZ* reporter gene under control of its endogenous promoter in strains PAO1 and PAO1Δ*hfq*. Our results indicated that Hfq does not have a significant impact on the

translation of *rpoN* (Fig. 28A). The observed low β-galactosidase activities could suggest that RpoN is autoregulated (Köhler *et al.,* 1994). Therefore, we decided to assess translation of the *rpoN*::*lacZ* fusion gene under the control of the P_{tac} promoter. Our results indicated that Hfq does not have a significant impact on the expression of RpoN. We observed, that direct regulation of *rpoN* translation by Hfq does not occur (Fig. 28B). This was also observed under conditions closer to physiology, *i.e.* when the strains were grown in minimal medium (Fig. 28C).

We conclude that Hfq does not regulate *anr* expression *via* RpoN, and therefore the molecular mechanism by which Hfq-mediated regulation of Anr occurs remains elusive. However, previous studies in our group have shown that the stringent response up-regulates *anr* transcription. It was shown that the transcription of *anr* was significantly reduced during severe nutrient starvation in a Δ*relA*Δ*spoT* double mutant when compared

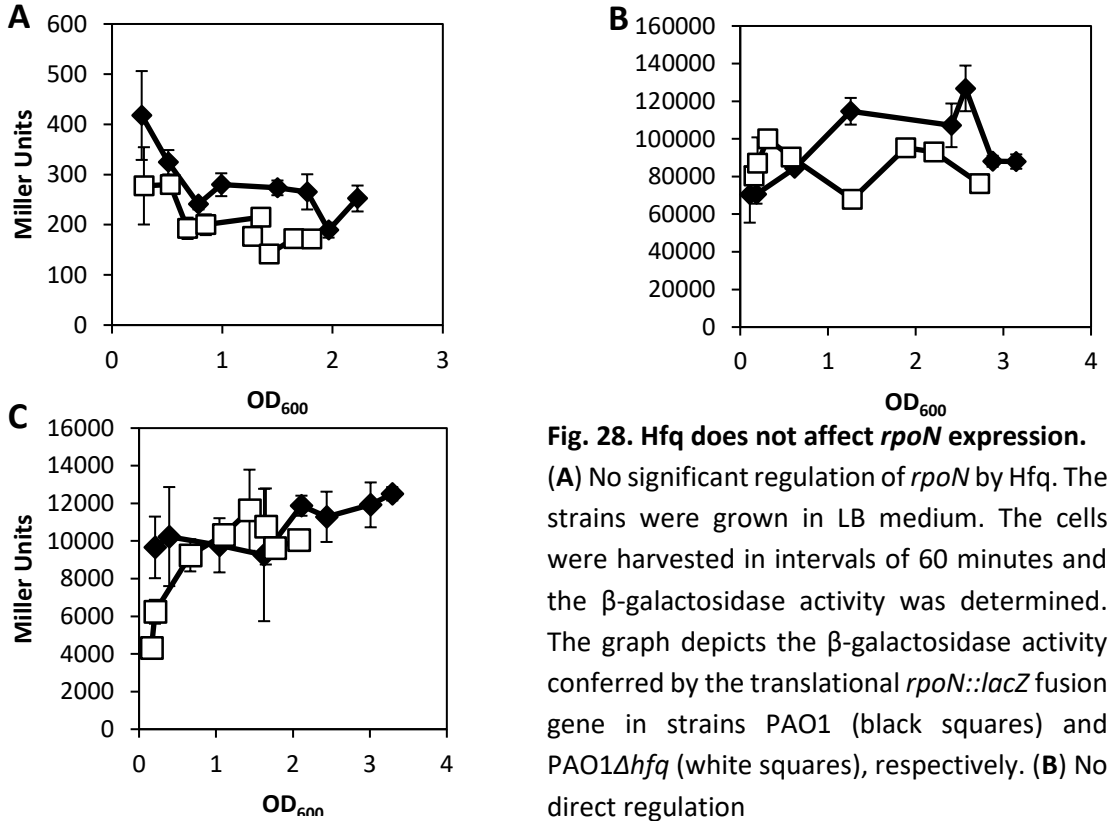

Fig. 28. Hfq does not affect *rpoN* expression. (**A**) No significant regulation of *rpoN* by Hfq. The strains were grown in LB medium. The cells were harvested in intervals of 60 minutes and the β-galactosidase activity was determined. The graph depicts the β-galactosidase activity conferred by the translational *rpoN::lacZ* fusion gene in strains PAO1 (black squares) and PAO1*Δhfq* (white squares), respectively. (**B**) No direct regulation of *rpoN* translation by Hfq. The strains were grown in LB medium. The cells were harvested in intervals of 60 minutes and the β-galactosidase activity was determined. The graph depicts the β-galactosidase activity conferred by the P_{tac} controlled translational *rpoN::lacZ* fusion in strains PAO1 (black squares) and PAO1*Δhfq* (white squares), respectively. (**C**) No regulatory effect of Hfq on *rpoN*::*lacZ* translation in minimal medium. The strains were grown in BSM medium supplemented with 40 mM of succinate. The cells were harvested in intervals of 60 minutes and the β-galactosidase activity was determined. The graph depicts the β-galactosidase activity conferred by the translational *rpoN::lacZ* fusion in strains PAO1 (black squares) and PAO1*Δhfq* (white squares), respectively. The error bars depict standard deviations of three independent experiments.

to a Δ*relA* strain (K. Kavalchuk, unpublished). The transcription of *anr* might be indirectly activated by the stringent response. Under conditions inducing the stringent response, ppGpp inhibits transcription of ribosomal genes from RpoD-dependent promoters. This leads to the generation of a pool of free core-RNAP, which becomes available for binding to alternative sigma factors. This could activate the transcription of RpoN-dependent genes, including *anr*.

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5. Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen belonging to the family of γ-proteobacteria and causes a variety of infections, especially in immunocompromised patients. The RNA chaperone Hfq regulates both virulence and metabolism in concert with small regulatory RNAs.

The regulatory RNA CrcZ is upregulated during alleviation of carbon catabolite repression (CCR) in *P. aeruginosa* and is able to bind to and sequester Hfq. As CrcZ was shown to bind to Hfq with high affinity, we therefore hypothesized that CrcZ interferes with canonical riboregulation by Hfq. In this study we first showed, that *P. aeruginosa* Hfq is required for canonical riboregulation of the *antR* gene by the sRNAs PrrF1-2. We further showed that ectopic over-expression of CrcZ results in a 1.5-fold decrease in repression of *antR* by the Hfq/PrrF entity. This may be explained by the sequestration of Hfq through the sRNA CrcZ. In support, we showed that *crcZ* over-expression de-represses translation of *algC* which is repressed by the sRNA ErsA. We further showed that under conditions of high levels of c´CrcZ (*e.g.* when the cells are grown in the presence mannitol) *antR* is de-repressed up to 6-fold, while the transcript levels of *antR* remained unchanged. Finally, we showed that a PAO1Δ*crcZ* strain is unable to utilize anthranilate as an alternative C-source and that a Δ*hfq* strain is unable to utilize both, succinate and anthranilate, in a hierarchical manner. Therefore, we concluded that the small RNA CrcZ can interfere with canonical riboregulation.

The anaerobic transcriptional regulator Anr is required by *P. aeruginosa* for regulation of anaerobic respiration, virulence and metabolism. Previous studies revealed that 20 out of 36 genes controlled by Anr regulon overlap with the Hfq regulon. In addition, it was shown that Hfq up-regulates *anr* expression. Therefore, the second part of this study was devoted to the regulation of *anr*. Here, we showed that efficient transcription of *anr* depends on the sigma factor RpoN. The growth of a *rpoN* mutant strain was severely attenuated during anaerobiosis, which was attributed to a 2-fold decrease in *anr* transcription. One possibility was that Hfq up-regulates *anr* transcription *via* up-regulation of *rpoN*. However, the results of this study showed that the translation of *rpoN* is not significantly changed by Hfq. We therefore concluded that Hfq does not up-regulate *anr via* RpoN.

6. Zusammenfassung

Pseudomonas aeruginosa ist ein opportunistisches humanpathogenes Bakterium, welches vor allem in immunsupprimierten Personen eine Vielzahl an Infektionen hervorruft. Das RNA Chaperone Hfq reguliert sowohl die Virulenz, als auch den Metabolismus von *P. aeruginosa* zusammen mit kleinen regulatorischen RNAs (sRNAs).

Die regulatorische RNA CrcZ wird nach Aufhebung der Kohlenstoff Katabolit Repression exprimiert und kann freies Hfq Protein binden. Aufgrund der hohen Affinität von CrcZ zu Hfq haben wir vermutet, dass CrcZ in der Riboregulation durch Hfq interferieren kann. In dieser Studie konnten wir zeigen, dass Hfq zusammen mit der regulatorischen RNA PrrF für die Repression von *antR* notwendig ist. Weiters konnte gezeigt werden, dass die ektopische Überexpression von *crcZ* die Inhibition der Translation von *antR* durch den Hfq/PrrF Komplex um einen Faktor von bis zu 1.5 verringert. Das kann durch die Sequestration von Hfq durch CrcZ erklärt werden. Dadurch ist weniger freies Hfq für die Regulation anderer Gene durch sRNAs vorhanden. Übereinstimmende Ergebnisse konnten auch bei der Regulation des Gens *algC* beobachtet werden, dessen Translation durch Hfq und die regulatorische RNA ErsA reprimiert wird. Auch hier konnte bei der *crcZ* Überexpression eine De-repression von *algC* gezeigt werden. Unsere Hypothese, dass CrcZ mit der Riboregulation interferiert konnte auch unter Bedingungen von physiologisch-bedingt höheren CrcZ Konzentrationen bestätigt werden. So konnte unter diesen Bedingungen ein bis zu 6-facher Anstieg der *antR* Translation beobachtet werden, während die Transkription nicht beeinflusst war. Schließlich zeigen wir, dass die Nutzung der alternativen Kohlenstoffquelle Anthranilat in einem PAO1Δ*crcZ* Stamm inhibiert ist und ein PAO1Δ*hfq* Stamm keine hierarchische Nutzung von alternativen Kohlenstoffquellen zeigt.

Der anaerobe Transkriptionsfaktor Anr reguliert in *P. aeruginosa* die Virulenz, die anaerobe Atmung und den Metabolismus. Vorangehende Studien haben gezeigt, dass 20 von 36 Genen des Anr Regulons auch von Hfq reguliert werden und der Sigmafaktor RpoN, sowie der Transkriptionsfaktor NtrC wichtig für die Transkription von *anr* sind. Zusätzlich ist bekannt, dass die Expression von *anr* durch Hfq aktiviert wird. Deshalb befasst sich der zweite Teil dieser Studie mit der Aufklärung dieser Regulation. Hier konnten wir zeigen, dass der Sigmafaktor RpoN die effiziente Transkription von *anr* bewirkt, aber nicht essentiell ist. Weiters wurde gezeigt, dass das Wachstum eines Δ*rpoN* Stamms unter anaeroben Bedingungen stark inhibiert ist, was wir auf eine 2-fache Reduktion in der Translation von *rpoN* zurückführen. Aus diesem Grund wurde vermutet, dass Hfq die Transkription von *anr* direkt oder indirekt über RpoN aktiviert. Jedoch zeigten unsere Ergebnisse, dass Hfq keinen Einfluss auf die Expression von *rpoN* hat. Daher kann die Aktivierung der *anr* Expression durch Hfq nicht durch RpoN bedingt sein.

7. Appendix

7.1 Solutions

7.1.1. Culture media

BSM medium:

K_2HPO_4	30.8 mM
KH_2PO_4	19.3 mM
$(NH_4)_2SO_4$	15 mM
$MgCl_2$	1 mM
$FeSO_4$	2 µM
C-source	40 mM

LB medium:

Peptone	10 g/l
NaCl	10 g/l
Yeast-extract	5 g/l

Adjust the pH to 7

If needed the medium was solidified by the addition of 15 g/l agar.

7.1.2. Buffers and solutions

7.1.2.1. Solutions for DNA

6x DNA loading dye (1ml):

Glycerol	50%
Bromphenol blue	2%
Xylene cyanol	2%
0,5M EDTA pH8	10 µl

Fill up to 1 ml

10xTBE:

Tris-base	892 mM
Boric acid	890 mM
EDTA	20 mM

Adjust pH to 8 with boric acid

6% PAA gel:

40% PAA (38:2)	30 ml
10xTBE	20 ml
ddH_2O	150 ml

Polymerize with 1/100th of 10% APS and 1/1000th TEMED

7.1.2.2. RNA isolation

RNA protection solution:

Phenol (saturated with H_2O)	5%

In 96% ethanol

DEPC-treated water:

DEPC	0.1%

After addition solution was vigorously mixed and left over night before autoclaving

Lysis buffer:

SDS	2%
Sodium acetate pH 5.5	30 mM
EDTA pH 8	3 mM

Dissolve in DEPC-treated water

Sodium acetate:

Sodium acetate	3 M

Dissolve in DEPC-treated water, adjust pH to 5.5 and autoclave

2X RNA loading dye (1ml):

Formamide	940 µl
10x TBE	40 µl
Xylene cyanol	2%
Bromphenol blue	2%

20% SDS:

20% SDS in DEPC-treated water

Hybridization solution:

2% BSA	5 ml
2% PVA	5 ml
2% FicoIII	5 ml
0.5M EDTA pH8	5 ml
20% SSC	62.5 ml
20% SDS	2.5 ml

Fill to 250 ml with DEPC-treated water
Fresh before use 2 g dextran and 200 µl salmon sperm DNA (10 mg/ml) were added to 20 ml of solution

20x SSC:

NaCl	3 M
Sodium citrate	300 mM

Adjust the pH to 7

Wash buffer I:

20x SSC	100 ml
20% SDS	5 ml

Fill to 1l with DEPC-treated water

Wash buffer II:

20x SSC	5 ml
20% SDS	5 ml

Fill to 1 l with DEPC-treated water

Methylene blue:

0.04% methylene blue in 0.5 M sodium acetate pH 5.5

7.1.2.3. β-galactosidase assay

Z-buffer:

Na_2HPO_4	60 mM
NaH_2PO_4	40 mM
KCl	10 mM
$MgSO_4$	1 mM
β-mercaptoethanol	0.27%

Add β-mercaptoethanol fresh before use

ONPG:

4 mg/ml ONPG in H_2O

Stop solution:

Na_2CO_3	1 M

7.1.2.4. Western Blot

2x SDS loading dye:

Tris/HCl	250 mM
SDS	6%
glycerol	20%
β-mercaptoethanol	10%

SDS running buffer:

Tris/HCl	3 g
glycine	200 mM
SDS	0.1%

Fill to 1 l with water, add SDS fresh

Lower Tris:

Tris/HCl	1.5 M
SDS	0.4%

Adjust pH to 8.8 with HCl

Upper Tris:

Tris/HCl	0.5 M
SDS	0.4%

Adjust pH to 6.8 with HCl

Transfer buffer:

Glycine	2,9 g
Tris/HCl	5.8 g
20% SDS	1.85 ml
Methanol	200 ml

Fill to 1 l and adjust pH to 8.3

10x TBS:

NaCl	80 g
KCl	2 g
Tris/HCl	30 g

Fill to 1 l with water and adjust pH to 7.5

Blocking solution:

5% skim milk in 1x TBS

Antibody solution (1:10 000):

Antibody	2 µl
1x TBS	19 ml
10% BSA	1.2 ml

Alkaline phosphatase buffer:

NaCl	5.8 g
$MgCl_2$	1 g
Tris/HCl	12 g

Fill to 1 l with water and adjust pH to 9.5

NBT/BCIP solution:

Alkaline phosphatate buffer	10 ml
0.5g NBT in 70% DMF	66 µl
0.5g BCIP in 100% DMF	33 µl

7.2. Abbreviations

Abbreviation	Meaning	Page
2-ABA	2-aminobenzoylacetate	4
2-HABA	2-hydroxyaminobenzoylacetate	4
AA	anthranilic acid	2
AI	autoinducer	2
asRNA	cis-encoded basepairing RNA	16
BSM	basal salt medium	30
C4-HSL	N-butyryl-homoserine lactone	4
CCR	Carbon catabolite repression	16
EBP	enhancer binding protein	8
Eσ^{70}	sigma 70 bound RNA polymerase holoenzyme	8
HHQ	2-heptyl-4-quinolone	4
HQNO	2-heptyl-4-hydroxyquinoline-N-oxide	4
HSL	homoserine lactone	3
PQS	Pseudomonas Quinolone Signal	2
QS	Quorum sensing	2
RBS	Ribosome binding site	16
RNAP	RNA polymerase	6
SD	Shine-Dalgarno sequence	14
TCA cycle	Tri carboxylic acid cycle	21
uof	upstream open reading frame	19
UTR	untranslated region	15

7.3. List of Figures