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MASTERARBEIT

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„Studies on the regulatory mechanisms of the sRNAs PaeII and PaeIII in *Pseudomonas aeruginosa* “

verfasst von

Stephan Rindler, BSc

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Betreut von:	Univ.- Prof. Dr. Udo Bläsi

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1. Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (Fig.1) is a rod shaped, Gram-negative, aerobic, motile, bacterium, belonging to the family of *Pseudomonaceae*.

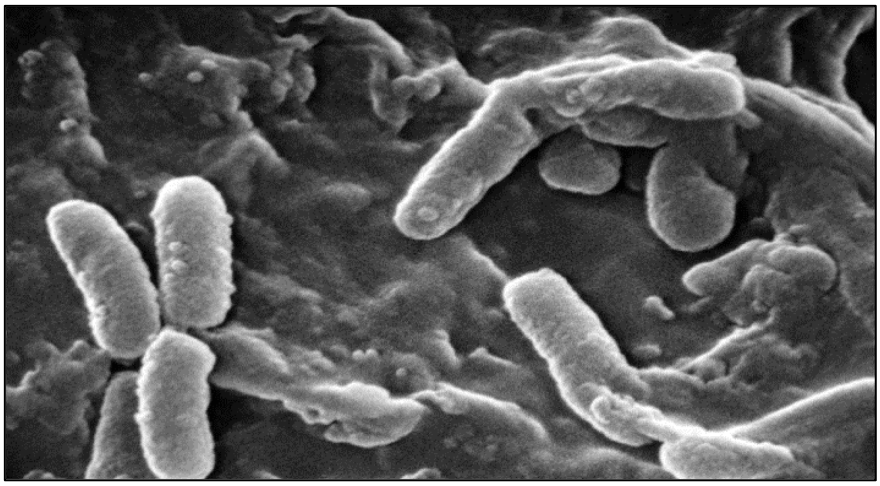

Fig.1 Scanning electron micrograph of *P. aeruginosa*.
(http://phil.cdc.gov/PHIL_Images/09232002/00005/PHIL_232_lores.jpg)

The ability to utilize a broad range of organic compounds enables *P. aeruginosa* to colonize almost any environment, from water and soil to plant, animal and human tissue. Having a large, 6.3 Mbp, dynamic genome allows *P. aeruginosa* to customize its genomic features, to fit the needs of almost any given ecological niche (Mathee *et al.*, 2007). Approximately 10% of all *P. aeruginosa* genes are contributing to its pathogenicity or are linked to biofilm formation (van Delden and Iglewski, 1998). Being an opportunistic human pathogen, *P. aeruginosa* rarely causes community-acquired infections in immunocompetent patients. However, it is the most common Gram–negative bacterium associated with nosocomial (hospital acquired) infections, causing high morbidity and mortality. Especially patients suffering from cystic fibrosis, cancer, AIDS and severe burns are subject to *P. aeruginosa* infections. The bacterium causes various disorders: infections of bones and joints, respiratory system infections, urinary and gastrointestinal tract infections, bacteremia and dermatitis (van Delden and Iglewski, 1998, Kerr and Snelling, 2009).

Such diverse Infections require a large number of various both, cell-associated (flagellum, pilus, nonpilus adhesins, alginate, lipopolysaccharide) and extracellular (hemolysins,

exotoxin A, exoenzyme S, proteases, pyocyanin, and hydrogen cyanide) virulence factors.

The course of diseases caused by *P. aeruginosa* can be divided into different phases.

In the initial phase bacteria need to breach the first line of defenses. Normally, mucosal and cutanenous barriers as well as the protective mucosal flora would prevent *P. aeruginosa* from entering the body. Breaks of these barriers after surgery, trauma, or treatment using broad-spectrum antibiotics can therefore enable *P. aeruginosa* to colonize damaged epithelia (van Delden and Iglewski, 1998). During this initial phase mostly cell-associated virulence factors are needed. Among several surface associated adherence factors and adhesins, mainly the type–4 pilus is responsible for *P. aeruginosa* attachment (Hahn and Heinz, 1997). Additionally, flagella, which are mainly responsible for mobility, can also act as adhesins (Feldman *et al.,* 1998). Flagellin, the major flagellar protein, is responsible for adhesion of *P. aeruginosa* to Muc1 mucin, which is a glycoprotein on the epithelial cell surface (Lillehoj *et al.,* 2001).

After colonization, high amounts of several extracellular virulence factors are produced which cause extensive tissue damage, allowing further dissemination through the bloodstream. Consequently, this leads to an acute infection (van Delden and Iglewski, 1998). The most prominent virulence factors include Exotoxin A which is produced by most *P. aeruginosa* strains involved in clinical infections. Like diphtheria toxin it inhibits protein synthesis and ultimately provokes cell death (Wick *et al*., 1990). Therefore, Exotoxin A is mainly responsible for local tissue damage which leads to bacterial invasion (Woods and Iglewski, 1983). Moreover, a study by Vidal *et al*. (1993) suggests that Exotoxin A is an immunosuppressive agent.

Exotoxin S causes tissue damage during lung infections, thereby possibly enhances bacterial dissemination (Nicas *et al*., 1985). By inhibiting wound healing and tissue regeneration Exotoxin S may contribute to maintain sites of colonization (Barbieri, 2000).

P. aeruginosa produces two hemolysins, Phospholipase C, a heat-lablile haemolysin and rhamnolipids (Berka *et al.,* 1982; Maier *et al.,* 2000). Together, these factors enhance spreading of the bacterial infection by breaking down lecithin and lipids (van Delden and Iglewski, 1998).

The lungs are protected by a surfactant consisting of phospholipids. Rhamnolipids show a detergent-like structure and are able to solubilize the phospholipids of the lung surfactant (Liu, 1974). Several proteases including LasA, LasB and alkaline protease are responsible for

cleavage of numerous host proteins such as elastin, collagen and proteins of the innate immune system (Toder *et al.,* 1994; van Delden and Iglewski, 1998).
Surprisingly, despite the wide spectrum of potential hosts, *P. aeuruginosa* possesses only four effector proteins (ExoS, ExoT, ExoU, and ExoY) which are directly injected into host cells (Hauser, 2009).

Pathogens have evolved many strategies to evade the host´s immune system and to withstand therapeutic intervention. *P. aeruginosa* disposes several resistance mechanisms, including production of antibiotic modifying enzymes and multidrug efflux pumps (Nakae *et al.*, 1995; Nikaido *et al.*, 1996; Piddock *et al.*, 2006). The major pump in wild type cells is the MexAB-OprM efflux system, which is able to excrete a variety of different drugs. Further, the bacterium possesses a highly impermeable outer membrane (Li *et al.,* 2000). Also, the ability to form biofilms and the production of the exopolysaccharide alginate additionally aggravates clearance of *P. aeruginosa* (Davies *et al.,* 1998).

The course of a *Pseudomonas* infection can develop into an acute or into a chronic disease. The switch between persistent and acute infection is mediated by a two component system including two small RNAs RsmY and RsmZ and the translational repressor protein RsmA (Goodman *et al.*, 2004). During an acute infection high amounts of extracellular virulence factors are produced. Tissue damage, blood vessel invasion, extensive inflammations cause multiple organ failure, which ultimately leads to death of the patient. In contrast, during chronic infections only low levels of extracellular virulence factors are secreted. Tissue damage is mainly caused by chronic inflammations (van Delden and Iglewski, 1998). Chronic lung infections by *P. aeuriuginosa* appear mainly in individuals suffering from cystic fibrosis. This inherited, recessive and autosomal disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Although several organs are affected, the most characteristic effects of the disease concern the respiratory system. Thick mucus accumulates in the lungs, where it serves as breeding ground for bacteria, especially for *P. aeruginosa*, which is the main pathogen that leads to chronic cystic fibrosis lung disease. During the initial infection, a planktonic, non–mucoid *P. aeruginosa* strain penetrates the mucus using its flagellum. An untreated initial infection may evolve into a chronic infection involving mucoid *Pseudomonas* strains. After a biofilm has formed through synthesis of

exopolysaccharides, the infection cannot be eradicated anymore and *P. aeruginosa* will persist in the patient's lungs for years. Therapy involves a combination of different antibiotics, which at least improves the quality of life in cystic fibrosis patients (Gaspar *et al.*, 2013).

1.2 Small regulatory RNAs in Bacteria

Being able to successfully adapt to different environments and to respond towards stress conditions is important for bacterial survival. It requires adaption of the bacterial physiology, which is caused by the expression of different sets of genes. The majority of studied sRNAs are known to be responsible for the regulation of gene expression. Modulation of transcription and translation, changes in mRNA stability, DNA maintenance or silencing are caused by various molecular mechanisms such as changing RNA conformation, protein binding, base-pairing with other RNAs and interactions with DNA. Base-pairing sRNAs can either be encoded in *cis* or *trans*. Those encoded in *trans* frequently require the RNA chaperone Hfq for function (Waters and Storz, 2009).

1.2.1 *Cis*-encoded base-pairing antisense RNAs

Cis-encoded antisense RNAs are transcribed from the DNA strand opposite to their target (Fig.2). Hence, they are fully complementary to their target RNA. They usually are between 50 and 300 nucleotides long and contain one to four stem loops. The minority of antisense RNAs are chromosomally encoded. Several of them are encoded on plasmids, within transposons and are found in the genome of bacteriophages. They often secure the correct copy number of the respective mobile element.

Fundamental processes such as conjugation, initiation of replication, suicide, mRNA degradation and translation initiation are regulated by *cis*-endcoded RNAs. For proper copy number control, the inhibitor *cis* antisense RNA must be in excess over the target. Therefore, plasmid-encoded antisense RNAs are expressed constitutively and have a short half-life. In contrast, chromosomally encoded *cis*-acting RNAs are only expressed under certain conditions and are more stable (Brantl, 2002; Vogel, 2003; Brantl, 2007). The post

transcriptional regulatory mechanisms caused by antisense RNAs are mostly inhibitory and only a few mechanisms of mRNA activation have been reported.

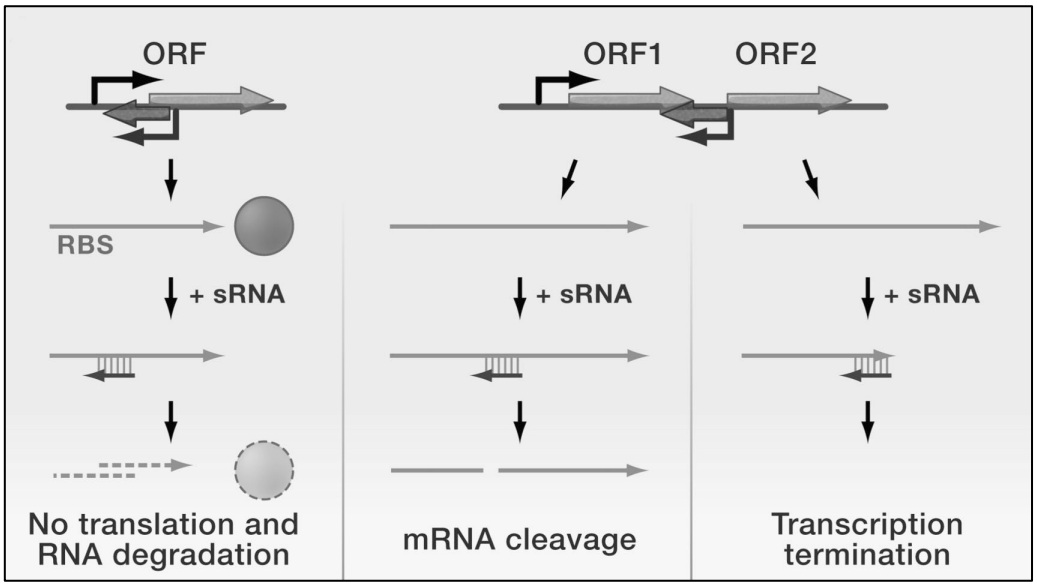

Fig.2 *Cis*–acting RNAs have different modes of action. Base-pairing of the RNA to its target mRNA can inhibit ribosome binding and often leads to mRNA degradation (left). An antisense RNA encoded between two genes of an operon can after base-pairing to the mRNA induce RNase cleavage (middle) or transcriptional termination (right) (modified from Waters and Storz, 2009).

The underlying molecular mechanisms of those regulatory effects include transcription attenuation, translation inhibition, inhibition of primer maturation, prevention of pseudoknot formation and either promotion or inhibition of mRNA degradation (Fig.2) (Brantl, 2007).

1.2.2 *Trans*-encoded base-pairing sRNAs

The second group of base-pairing sRNAs is transcribed in *trans* (Fig.3). In contrast to antisense RNAs they share only limited complementarity with target mRNAs, which often enables base-pairing with several mRNAs. Even if there are longer complementary regions, only a distinctive binding site is required for regulation. Most of the characterized *trans*-encoded sRNAs bind to the 5' UTR of mRNAs (Gottesman, 2005; Kawamoto *et al*., 2006; reviewed in: Aiba, 2007, Waters and Storz, 2009).

The majority of known *trans*-encoded sRNAs are negative regulators. Binding of a sRNA near the ribosome binding site (RBS) of the target mRNA can block translation. Base-pairing can

also occur distant of the RBS which affects the stability of the target mRNA. Both modes of action can lead to RNA degradation by RNase E (De Lay, *et al.,* 2013).

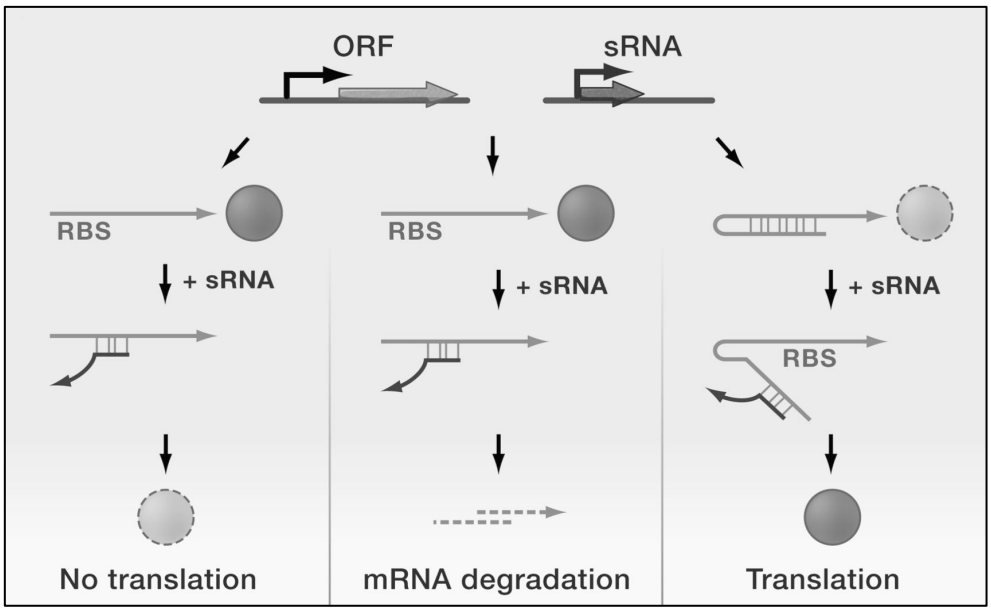

Fig.3 Regulatory functions of *trans*–encoded sRNAs. Base-pairing of sRNA and mRNA can block ribosome binding (left). Association of sRNA and mRNA generates a duplex, which is degraded by RNases (middle). By binding to and by removal of inhibitory secondary structures translation is enabled (right) (modified from Waters and Storz, 2009)

However, also gene activation by *trans*–encoded sRNAs has been reported. Binding of a sRNA in an anti-antisense manner prevents formation of, or removes inhibitory secondary mRNA structures. Consequently, ribosomes association and translation are enabled (Repoila *et al.*, 2003; Waters and Storz, 2009). One example is the *E. coli* sRNA DsrA. It base-pairs with the *rpoS* mRNA encoding the stationary phase sigma factor σ^S and relieves an inhibitory secondary structure, which sequesters the ribosome binding site of the long *rpoS* transcript. Therefore, binding of the sRNA DsrA to *rpoS* mRNA leads to increased translation (Storz *et al.,* 2004; Gottesman, 2005).

Successful regulation by *trans*–encoded sRNAs frequently requires Hfq, an RNA binding protein of the SM-like protein family. It promotes base-pairing either by structural changes of either RNA, or by increasing the local concentrations of both binding partners. Also, Hfq can protect RNAs from being degraded and it can act as an RNA chaperone to open up folded structures for interaction (Gerhart and Wagner, 2013).

1.2.3 The RNA chaperone Hfq

The Hfq protein (reviewed in Brennan and Link, 2007; Vogel and Luisi, 2011) was first identified in *E. coli* as a host factor needed for replication of the RNA bacteriophage Q in the late 1960s. In 2002, structural and bioinformatic studies performed by Zhang *et al.* (2002) revealed Hfq as a member of the SM- and SM-like protein family. A phylogenetic study performed by Sun *et al.* (2002) detected Hfq orthologues in many Gram-positive and Gram-negative bacteria. Sm- and Sm-like proteins are also present in eukaryotes and archaea. Eukaryotic Sm- proteins serve as scaffolds for ribonucleoproteins, whereas the function of archael SM- proteins remains unclear (Mura *et al.,* 2013). However, in *Methanococcus jannaschii* an "Hfq-like" protein was discovered, which could partially complement an *hfq* deletion in *E. coli* (Nielsen *et al.,* 2007).
Members of the Sm- and Sm-like protein family are alike in structure and function. They have a ring-like, multimeric quaternary architecture and are able to bind to RNAs and thereby contribute to post-transcriptional regulation (Wilusz and Wilusz, 2005).

The Hfq family of proteins forms thermostable homo-hexamers and their length ranges from 70 to 110 amino acid residues. Hfq is highly abundant in *E. coli* (~10000 hexamers per cell) (Brennan and Link, 2007). *E. coli hfq*$^{-}$ mutants show pleitropic effects such as: decreased growth rates, increased sensitivity to UV light, mutagens and antioxidants, as well as increased oxidation of carbon sources (Tsui *et al.,* 1994). The ring-like structure of Hfq features sites for interaction with nucleic acids at the proximal and distal site of the hexamer. However, each face recognizes a different binding motif (Vogel and Luisi, 2011). Co-crystallization of *Staphylococcus aureus* Hfq with single stranded U-rich sequences showed that they are bound at the proximal face of Hfq. The RNA is bound in a circular manner around the highly basic inner pore of the Hfq hexamer (Schumacher *et al.,* 2002) (Fig.4, top).
The crystal structure of *E. coli* Hfq protein in complex with RNA showed, that the distal face interacts with poly(A) RNA (Fig.4, bottom). Preferentially, it binds sequences containing ARN or ARNN motifs, in which R is a purine and N is any base (Link *et al.,* 2009).

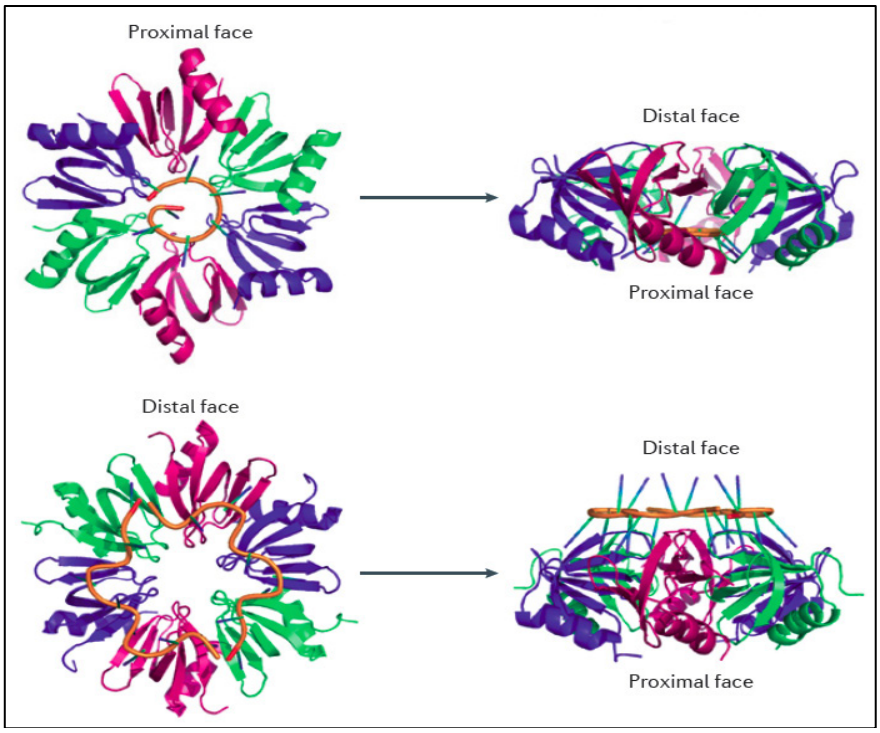

Fig.4 The structure of Hfq and its interactions with RNA. One unit of the Hfq hexamer is composed of one α-helix and five β-strands. The RNA is bound in a circular manner (orange) on the proximal (top) and distal (bottom) face of the ring formed by the Hfq hexamer (modified from Vogel and Luisi, 2011).

A model how Hfq simultaneously binds a sRNA and its target mRNA was first proposed by Mikulecky *et al.* (2004). Later, it could be shown that the distal face binds mRNA, whereas the proximal side binds the sRNA. However, some RNAs may also bind to either side (Fender *et al.,* 2010).

Hfq is widely accepted to be an important member of a global post-transcriptional network in which it is required for interaction of regulatory *trans*-encoded sRNAs with mRNAs (Vogel and Luisi, 2011). Several general mechanisms of Hfq-mediated gene regulation are known. Hfq facilitates binding of a sRNA to the 5' region of its target mRNA. Consequently, ribosome binding can be blocked and translation is inhibited (Fig.5a). Secondary structures formed by the mRNA can mask the ribosome binding site and thereby impede translation. Hfq may guide a sRNA in order to disrupt these secondary structures (Fig.5b). Also, Hfq can protect sRNAs from ribonuclease cleavage (Fig.5c) and it can promote mRNA cleavage (Fig.5d). Finally, Hfq can render 3' ends accessible for polyadenylation and subsequent 3'-to-5' exonucleolytic degradation, thereby it promotes RNA turnover (Fig.5e) (Vogel and Luisi, 2011).

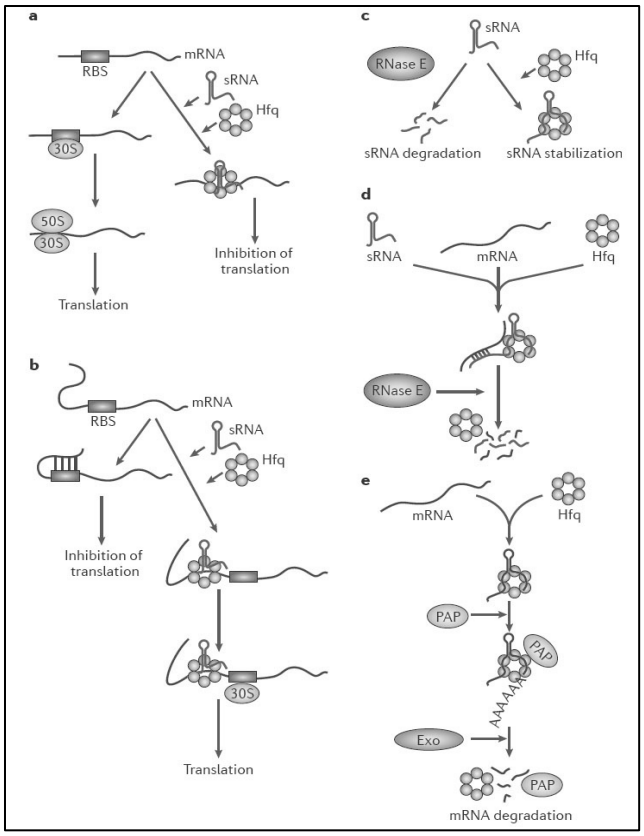

Fig.5 Modes of Hfq activity.
a. Hfq in concert with a sRNA can prevent ribosome binding
b. Secondary structures can mask the RBS, interaction of Hfq–sRNA complex may open these structures.
c. Hfq may protect sRNAs from RNase E cleavage, or
d. It induces cleavage of sRNA–mRNA duplexes by recruitment of RNase E.
e. Hfq may stimulate the polyadenylation of mRNA, which in turn triggers 3'-5' degradation by exoribunocleases
(modified from Vogel and Luisi, 2011).

Although the post transcriptional effects of Hfq action are well described, the molecular mechanisms of Hfq function are still unclear.

Sauer *et al.* (2012) identified additional RNA binding sites on the lateral surface of the ring formed by Hfq monomers, which can act independently and are distinct from the proximal and distal face. Therefore, they proposed a different model for RNA binding by Hfq where the sRNA is bound to the proximal site via its 3' U-rich terminator end. The body of the sRNA is wraped around the ring and serveral lateral binding sites protect it. Hence, a single Hfq hexamer can protect both the sRNA body and 3' end from degradation. The unbound 5' mRNA complementary region of the sRNA/Hfq complex can therefore target mRNAs which are consequently bound at the distal site (Sauer *et al.,* 2012) (Fig.6).

Furthermore, this new model of sRNA binding by Hfq also helped to understand how binding partners of Hfq are exchanged rapidly, although Hfq/RNA complexes are very stable. It led to the development of the active cycling model of RNAs on Hfq (Gerhart and Wagner, 2013).

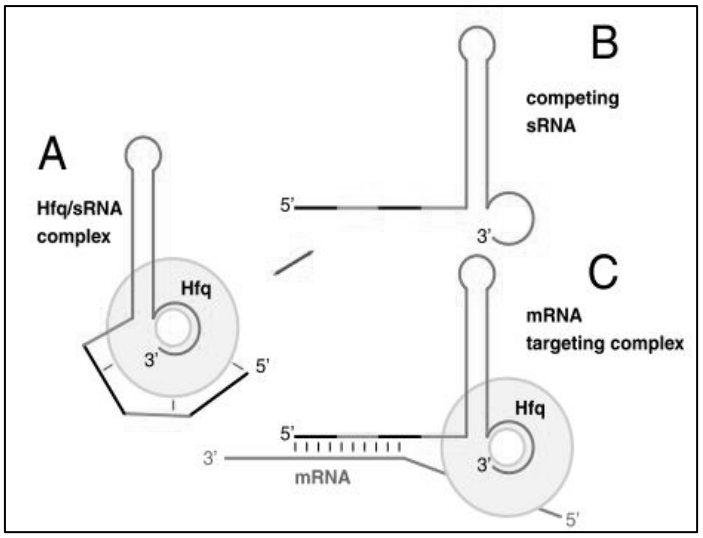

Fig.6 Model of sRNA interaction with hexameric Hfq.
a. The 3' end of the sRNA is bound to the proximal RNA binding site, the terminator hairpin is located above the proximal surface. The sRNA body is bound to several lateral RNA binding sites of Hfq.
b. Competing sRNAs can swiftly replace the bound sRNA molecule by replacement of lateral contacts.
c. The mRNA targeting complex, Hfq increases the local concentrations of two RNAs and thereby facilitates base pairing (Sauer *et al.,* 2012).

Within the cell, RNA ligands are present in excess to Hfq proteins, therefore the RNA-binding sites of Hfq are always fully occoupied *in vivo.* The model proposes that Hfq bound RNAs occupy several subunits of the lateral binding site. At first, a free competitor RNA contacts a single unoccoupied subunit which is followed by a series of reversible replacement steps where the "old" and "new" RNA species switch places (Fig.7). These steps do not require external energy, which is supported by the report of Hämmerle *et al.* (2012) that Hfq action is neither ATP-dependent, nor did they observe a proposed destabilizing effect of ATP on Hfq-RNA complexes. This model succeeded to explain how it is possible that the effects of newly induced sRNAs on target mRNAs are already detected after 1-2 minutes although RNA-Hfq complexes are very stable (Gerhart and Wagner, 2013).

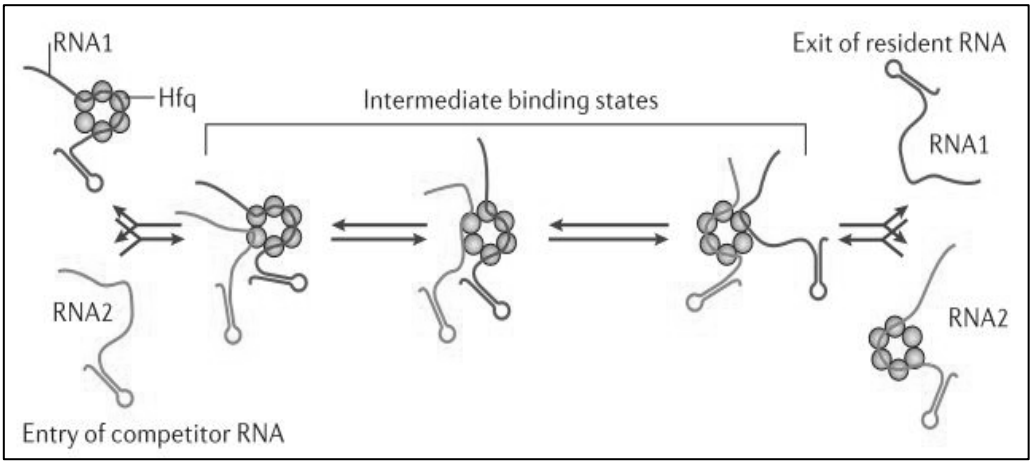

Fig.7 A model for RNA exchange on Hfq. A competitor RNA binds to an available lateral Hfq interaction site. In intermediate, reversible, binding steps the resident RNA is replaced gradually by the competitor RNA (Vogel and Luisi, 2011).

P. aeruginosa Hfq, like the *E. coli* ortholog, forms symmetric donut-shaped homo-hexamers, but its C-terminal tail is only 18 aa residues in length, whereas *E. coli* Hfq possesses a 38 residues long C-terminus (Nikulin *et al.,* 2005). The *Pseudomonas* Hfq can functionally replace *E. coli* Hfq and it is also required for *rpoS* expression in PAO1 (Sonnleitner *et al.,* 2002; Sonnleitner *et al.* 2003). A *Pseudomonas* transcriptome analysis by Sonnleitner *et al.* (2006) indicated that Hfq is involved in several different pathways. This includes effects on quorum sensing controlled genes which can explain the reduced virulence of a PAO1 *hfq*$^-$ strain (Sonnleitner *et al.*, 2003). In total Hfq affected approximately 5 % of the PAO1 transcriptome in an *rpoS* independent manner (Sonnleitner *et al.,* 2006). A recent study by Sonnleitner *et al.* (unpublished) suggested that *P. aeruginosa* Hfq acts as a translational repressor of transcripts subjected to carbon catabolite repression in PAO1.

1.2.4 sRNAs acting by protein sequestration

In addition to protein-binding sRNAs, which have either intrinsic activity (RNaseP) or are part of a ribonucleoprotein particle (4.5S and tmRNA), there are regulatory RNAs that sequester regulatory proteins, e.g. CsrA (reviewed in Babitzke and Romeo, 2007), or even RNA polymerase (reviewed in Wassarman, 2007) (Fig.8). RNA binding proteins recognize specific binding motifs displayed by their target RNA. As these binding motifs are imitated by protein binding RNAs, like CsrB, they can sequester these protein regulators. Consequently, the titration of free protein regulators can result in upregulation of their target mRNAs (Fig.8). In other words, RNAs which act by protein sequestration antagonize the activity of their respective target protein (Waters and Storz, 2009).

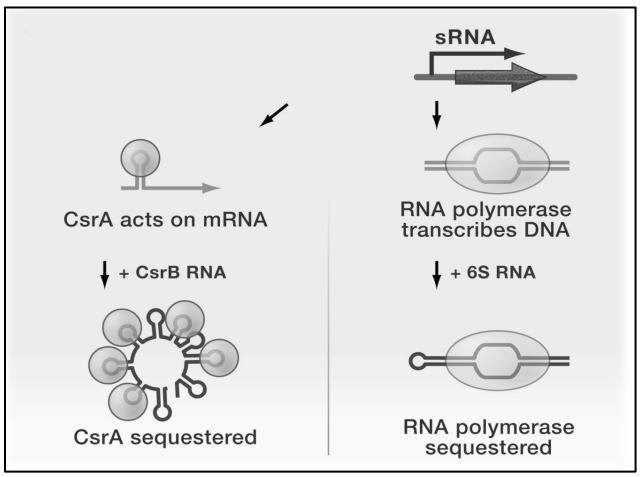

Fig.8 Protein binding sRNAs that antagonize regulatory proteins.
The RNAs CsrB and CsrC contain several CsrA binding motifs. They sequester CsrA and thereby prevent its function (left). High levels of 6S RNA lead to reduced transcription from σ^{70} dependent promoters (right).
(modified from Waters and Storz 2009).

CsrA is an RNA-binding protein that regulates carbon usage, motility, pathogenicity, quorum-sensing and response systems upon entry into stationary phase or during nutrient poor conditions. The CsrA dimer binds to GGA motifs in the 5' UTR region of their target mRNAs and thereby activates or represses mRNA expression post transcriptionally (Babitzke and Romeo, 2007; Romeo *et al.,* 2013). Two RNAs, CsrB and CsrC, antagonize CsrA action in *E. coli.* Both contain multiple GGA motif. CsrB shows 22 potential CsrA binding sites and is capable of sequestering ~9 CsrA protein dimers (Fig.8 left).
Thereby CsrB and CsrC indirectly activate or deactivate CsrA-dependent gene expression. Transcription of CsrB and CrsC sRNAs is dependent on the BarA-UvrY two-component system, which is activated under poor growth conditions (Suzuki *et al.*, 2002; Romeo *et al.,* 2013).

Bacterial promoter–directed transcription initiation requires the transcriptionally active core enzyme and a σ subunit, termed the holoenzyme, for promoter recognition. In *E. coli* 6S RNA competes for binding of σ^{70} RNA polymerase by mimicking a σ^{70} DNA promoter. The 6S RNA is transcribed as part of a bicistronic mRNA, which contains the gene encoding the 6S RNA (*ssrS)* and an open reading frame (*ygfA*) (Wassarman and Storz, 2000). Whether the σ^{70} RNA polymerase binds to DNA promoters or to 6S RNA is largely dependent on the levels of 6S RNA present in the cell. During exponential growth phase DNA promoters are predominately associated with σ^{70} RNA polymerase. The 6S RNA levels increase during growth, being highest in late stationary phase. Upon entry into stationary phase σ^{70} RNA polymerase binds mainly to the 6S RNA, which is available at high amounts. Therefore, σ^{70} dependent transcription is inhibited. The 6S RNA dependent changes in gene expression during stationary phase lead to increased long–term survival and render the cell resistant to stress conditions. If fresh, nutrient rich medium is provided the cells start to outgrow and consequently NTP levels are significantly elevated. 6S RNA not only binds σ^{70} RNA polymerase, it can also serve as a template for pRNA (product-RNA) synthesis if sufficient NTPs are present. Transcription of the pRNA leads to release and to degradation of 6S RNA (Wassarman and Karen, 2007) (Fig.8, right).

The synthesis of the metabolic enzyme glucosamine-6-phosphate (GLcN6P) synthase Glms is controlled by two homologous sRNAs GlmY and GlmZ and an the RNA binding adaptor protein RapZ. The synthesis of GlcN6P by GlmS is an important part of the amino sugar pathway, as the production of precursors for cell wall and outer membrane assembly depends on GlcN6P (Göpel *et al*., 2013).

GlmZ is a ~207nt long, base-pairing sRNA and its activating effect on *glmS* translation is Hfq dependent. The RNA chaperone Hfq mediates binding of full lenght GlmZ to an anti Shine and Dalgarno sequence in the *glmS* mRNA, which facilitates translation and enhances stability of the mRNA (Fig.9, right). However, GlmZ can be inactivated by processing to a 151nt variant, which lacks the *glmS* interaction site. The second sRNA GlmY, which is also processed by a yet unknown factor from a full length of 184nt to 147nt long variant, has an indirect activating effect on *glmS.* Upon decreasing of intracellular GLcN6P levels GlmY accumulates and inhibits processing of GlmZ. Thereby it indirectly enhances *glmS* translation (Fig.9, right).

The inhibitory factor in this metabolic process is a novel type of RNA binding adaptor protein former known as YhbJ. By binding to GlmZ the protein recruits RNase E, which leads to processing and inactivation of GlmZ. Therefore, YhbJ was renamed according to its mode of action: RapZ (RNase adaptor protein for sRNA GlmZ) (Fig.9, left). Since GlmZ and GlmY are highly similar in sequence and structure they compete for RapZ binding. If GlcN6P levels are low GlmY concentrations are high, the sRNA sequesters RapZ and consequently full length GlmZ is stabilized and able to stimulate *glmS* translation (Fig.9, right).

Additionally, it was shown in vitro that GlmZ is not an appropriate substrate for RNase E, processing requires the adaptor protein RapZ (Göpel *et al.,* 2013).

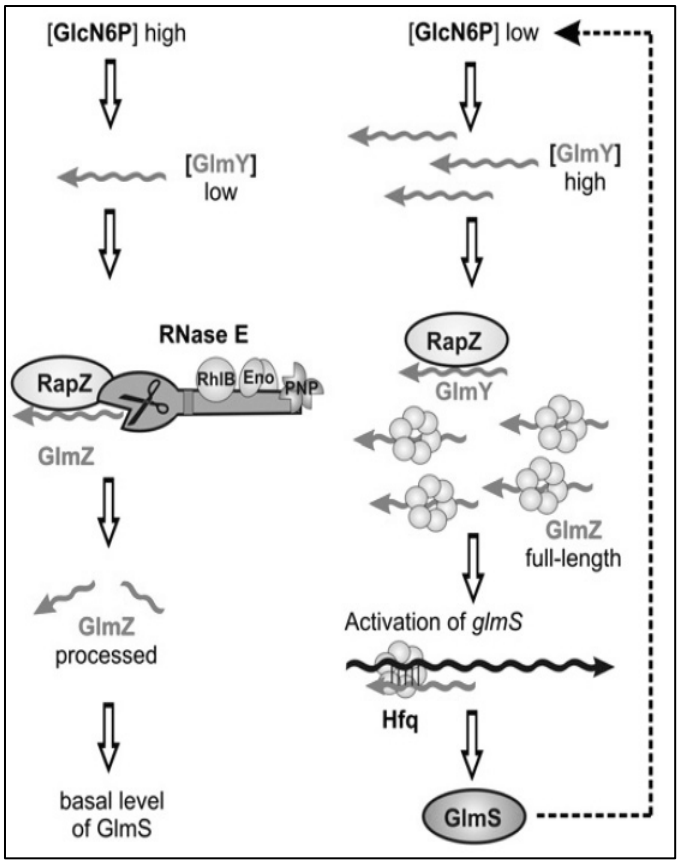

Fig.9 Model for GlmY/GlmZ dependent regulation of GlmS.
Under high GlcN6P concentrations, there are only low amounts of GlmY present in the cell. Therefore, the sRNA GlmZ, which would activate GlmS synthesis, is marked by RapZ for processing by RNase E and thereby inactivated (left).
Upon GlcN6P depletion, the GlmY concentration increases in the cell.
In consequence, RapZ is sequestered by GlmY and full length GlmZ accumulates. As a result, Hfq can mediate base-pairing of GlmZ to *glmS*, which enhances *glmS* translation (right). (modified from Göpel *et al.,* 2013).

1.2.5 DNA targeting sRNAs–CRISPR RNAs

This class of regulatory sRNAs is part of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system, which can be found in ~40% of Bacteria and ~90% of Archaea. These RNAs can confer resistance towards bacteriophages and can prevent conjugation of plasmids. Most CRISPR systems share a common architecture (Fig.10). The CAS (CRISPR-associated) genes are followed by an approximately 550bp long leader sequence. It is followed by a highly repetitive region composed of direct DNA elements (repeats), which are separated by similarly sized non-repetitive spacers. The spacers are derived from phages, integrated in the bacterial genome and provide resistance towards their respective associated bacteriophage. It is also shown that upon further phage infections new spacers can be integrated into this highly variable repeat-spacer region (Sorek *et al.*, 2008).

Fig.10 Typical structure of a CRISPR locus. See text (modified from Sorek *et al.,* 2008).

In a simplified model the repeats-spacer array is transcribed as a long RNA, which is recognized by CAS genes and processed to sRNAs, which contain a spacer and two half repeats. These sRNAs in complex with additional CAS proteins can target homologous DNA fragments and mark them for degradation (Sorek *et al.*, 2008) (Fig.11).

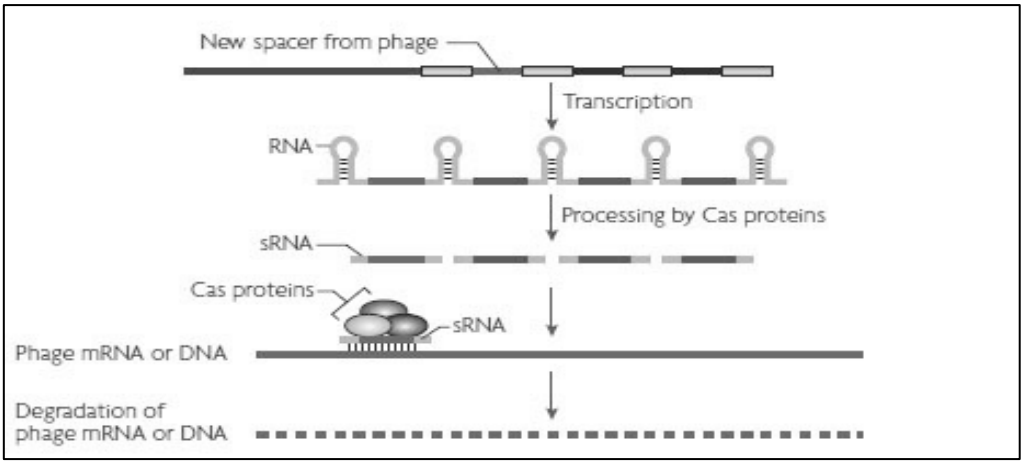

Fig.11 Simplified model for CRISPR action. The repeat-spacer array is transcribed as a whole, recognized and processed by CAS proteins to small RNAs. These sRNAs with the aid of Cas proteins base pair with phage nucleic acids and lead to their degradation (modified from Sorek *et al.,* 2008).

1.3 Small RNAs in *Pseudomonas aeruginosa*

Although *Pseudomonas spp*. have a large genome up to date only 40 sRNAs have been reported. All genomic surveys focused on intergenic regions. Therefore, these surveys did not account for *cis*-acting RNAs, which are encoded in open reading frames on the antisense strand (Sonnleitner *et al.,* 2011). Out of these 40 sRNAs the function of only a few has been revealed. In *Pseudomonas aeruginosa* sRNAs were reported to be involved in various metabolic processes including: catabolite repression, iron metabolism, quorum sensing and bacterial pathogenesis (Sonnleitner *et al.,* 2006; Sonnleitner *et al.,* 2010; Oglesby-Sherrouse and Vasil, 2010) (Table 1).

sRNA	Length (nucleotides)	Protein bound	Expression	Process regulated	Reference
PhrS	212	Hfq	Induced by low oxygen via ANR	Quorum sensing	Sonnleitner *et al.* (2011)
PrrF1	~110	Hfq	Induced by low iron via Fur derepression	Iron homeostasis	Wilderman *et al.* (2004)
PrrF2	~110	Hfq	Induced by low iron via Fur derepression	Iron homeostasis	Wilderman *et al.* (2004)
PrrH	~325		Repressed by iron and haem	Iron and haem homeostasis	Oglesby-Sherrouse and Vasil (2010)
RsmY	124	RsmA, Hfq	Induced by multiple signals via the GacS–GacA two-component system	Biofilm formation	Reviewed in Lapouge *et al.* (2008)
RsmZ	~120	RsmA	Induced by multiple signals via the GacS–GacA two-component system	Biofilm formation	Reviewed in Lapouge *et al.* (2008)
CrcZ	407		Induced in the presence of poor carbon sources via RpoN and the CbrA–CbrB two-component system	Catabolite repression	Sonnleitner *et al.* (2009)
RgsA	120		Directly regulated by RpoS and indirectly regulated by the GacS–GacA two-component system	Oxidative stress response?	González *et al.* (2008)
PhrD	72	Hfq			Sonnleitner *et al.* (2008)

Table 1 Small regulatory RNAs characterized in *P. aeruginosa.* (Sharma and Storz, 2011).

As mentioned above, there are different modes of sRNA action, all of which are present in *Pseudomonas*. Although *Pseudomonas* strains are strongly affected by *hfq* deletion and Hfq was shown to interact with sRNAs in co-immunoprecipitation assays, experimental proof for its involvement in sRNA-mRNA annealing is as yet lacking (Sonnleitner *et al.*, 2012). A study performed by Gómez-Lozano *et al.,* (2012) was one of the first to use RNA-sequencing to discover new sRNAs in *P. aeruginosa.* Over 500 novel sRNA candidates and a large set of potential regulatory molecules could be identified.

Morover, a RNA-sequencing study by Wurtzel *et al.* (2012) focused on the differential gene expression when *P. aeruginosa* is grown at 28°C or at the host related temperature of 37°C. In addition to dectecting temperature-induced genes, they identified 165 sRNAs and over 380 *cis*-antisense RNAs.

1.3.1 *Pseudomonas aeruginosa* RNAs acting by protein sequestration

Whether a *Pseudomonas* infection stays acute or becomes chronic depends to a large part on the GacS/A/RSM regulatory network (Fig.12).

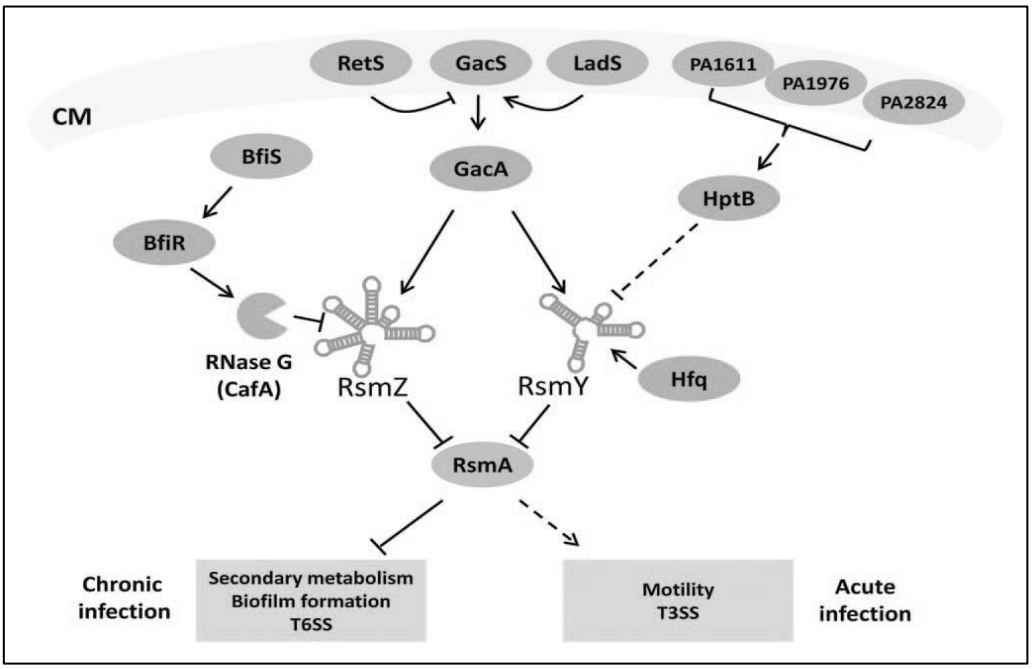

Fig.12 The Gac/Rsm regulatory network. Synthesis and stability of the RNAs involved is tightly regulated by several two-component systems, which are shown in the top part of the figure. This regulation allows the RNAs to respond to different environmental signals. The RNAs RsmZ and RsmY antagonize RsmA regulation and thereby mediate the switch between acute and chronic infections. ⊥, negative control; ↓, positive control; dashed lines, indirect control (Sonnleitner *et al.,* 2012).

The translational repressor protein RsmA belongs to the RsmA/CsrA protein family which regulates secondary metabolism and carbon storage. By binding to GGA motifs, which are often overlapping with the Shine and Dalgarno sequence, RsmA represses mRNA translation (Lapouge *et al.*, 2007; Schuber *et al.*, 2007). Among the down-regulated targets of RsmA are genes encoding proteins needed to establish a chronic infection, such as secondary metabolites, virulence factors (e.g. type VI secretion, extracellular enzymes) and carbon storage compounds**.** In contrast, genes required for acute infections (type III secretion, type IV pilus synthesis and flagellar motility) are indirectly up-regulated by RsmA.In addition, RsmA inhibits translation of regulatory factors, which are known to shut down genes needed for acute infection. Thereby RsmA helps to perpetuate the intense course of the disease (Brencic *et al.*, 2009; Sonnleitner *et al.,* 2012) (Fig.12).

The sRNAs RsmY and RsmZ

The two functional redundant RNAs RsmY and RsmZ allow the bacterium to adapt to the host environment by promoting the switch from an acute state to a chronic state of infection.

Upon activation of the GacS/GacA (global activation) two component system, transcription of both RNAs is mediated by the response regulator GacA (Brencic and McFarland *et al.*, 2009) (Fig.12, top). The synthesis of RsmY and of its target RsmA increases throughout growth, whereas *rsmZ* expression is observed mostly during late growth phase (Kay *et al.,* 2005). Although they share little sequence conservation RsmY and RsmZ are structurally conserved and both have several GGA motifs. RsmY possesses seven and RsmZ eight such motifs, which allow for RsmA binding and sequestration. Mostly, the GGA motifs are located in single stranded RNA regions such as loops or between stem loop structures (Valverde *et al.*, 2004).

The sequestration of RsmA by the RNAs leads to deregulation of translation of mRNAs which otherwise would be inhibited by RsmA.

The GacA/GacS system itself is controlled by two sensor kinases LadS and RetS, the latter of which blocks RsmZ transcription by direct interaction with GacS. As a consequence, genes for acute infection are induced (Ventre *et al.*, 2006; Goodman *et al.*, 2009). LadS is able to up-regulate *rsmZ* transcription through physical contact with GacS. Hence, it antagonizes RetS and promotes chronic infection (Sonnleitner *et al.*, 2012). In addition, the RNA chaperone Hfq also plays an important role in this regulatory network. It stabilizes RsmY (Sonnleitner *et al.,* 2006) and protects the RNA from RNase E cleavage *in vitro* (Sorger-Domenigg *et al*., 2007).

As shown in Fig.12, there are two additional mechanisms for modulation of RsmY/Z transcription. The histidine phosphotransferase protein B (HptB) negatively impacts *rsmY* expression (Hsu *et al.,* 2008).

The abundance of RsmZ was shown to be reduced in *P. aeruginosa* biofilms. A two component system, BfiSR, which is important in biofilms activates transcription of *cafA*. This gene encodes a protein that specifically cleaves RsmZ (Petrova and Sauer, 2010).

The RNA CrcZ

Another similar mechanism, involving the protein Hfq and the RNA CrcZ, contributes to carbon catabolite repression (Sonnleitner *et al.,* unpublished) (Fig.13).

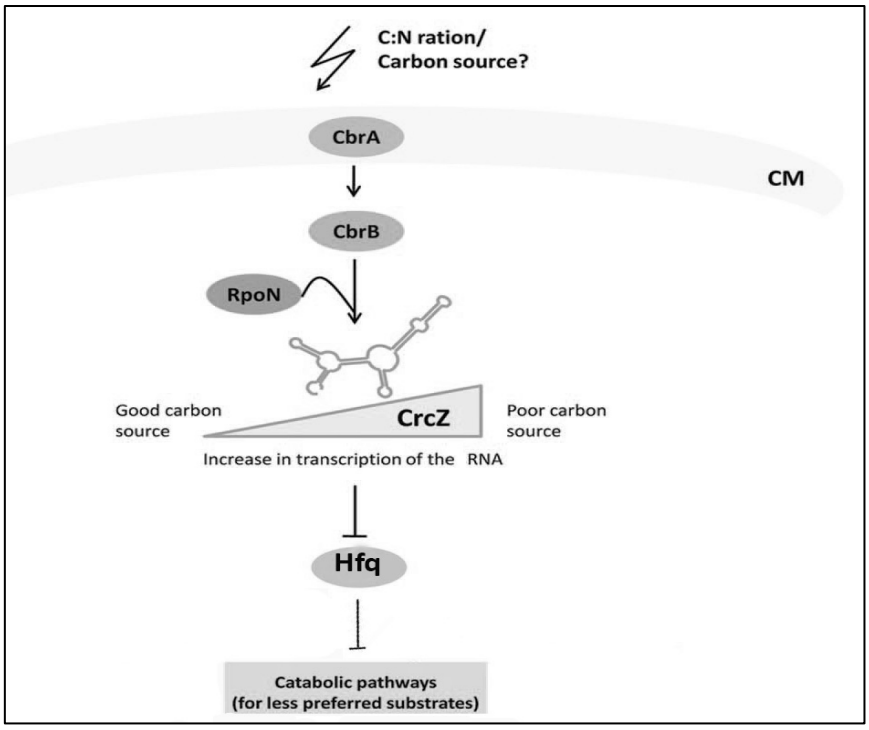

Fig.13 CrcZ–mediated regulation in *P.aeruginos*. CrcZ levels are dependent on the carbon source. In the presence of a poor carbon source (e.g., mannitol) the CrcZ levels are elevated and it can sequester Hfq. This in turn leads to activation of Hfq-repressed genes. The respective enzymes/proteins are needed for the utilization of less preferred substrates. ⊥, negative control; ↓, positive control; (Sonnleitner *et al.,* unpublished).

This mechanism allows bacteria to respond to available energy sources. Only genes which are needed for utilization of the preferred carbon source are expressed (Görke *et al.*, 2008; Rojo *et al.,* 2010). Similar to RsmA, Hfq binds to A-rich regions in the vicinity of the ribosome binding site on target mRNAs, thereby impairing translation. The RNA CrcZ contains A-rich binding sites and is able to sequester Hfq. The two component system CbrA/B (catabolic regulation two-component system) mediates *crcZ* expression in a σ^{54}-dependent manner. The activity of CbrA/B is dependent on the carbon source present, and thus is the CrcZ level. If there is a poor carbon source such as mannitol present, levels of CrcZ increase. As a result, CrcZ sequesters Hfq and consequently, Hfq-regulated genes are derepressed (Sonnleitner *et al.*, 2009).

1.3.2 *Pseudomonas aeruginosa* putative and verified base-pairing sRNAs

The sRNAs Prrf1, Prrf2 and Prrh

On the one hand Bacteria absolutely require iron as a cofactor of many enzymes. On the other hand excess of iron is toxic. Genes for iron acquisition in most Gram-negative Bacteria are negatively regulated by the ferric uptake regulator Fur. When complexed with its co-repressor Fe^{2+}, Fur binds to a target sequence (Fur box) in Fur dependent promoters (Hantke, 2001; Wilderman *et al.,* 2004). During iron starvation RhyB, an *E. coli* small RNA, negatively regulates genes which are involved in iron storage and which encode iron containing proteins. Binding of the sRNA near the ribosome binding site is facilitated by Hfq and impairs mRNA translation. The sRNA RhyB is negatively controlled by metallo-Fur (Massé and Gottesman, 2002).

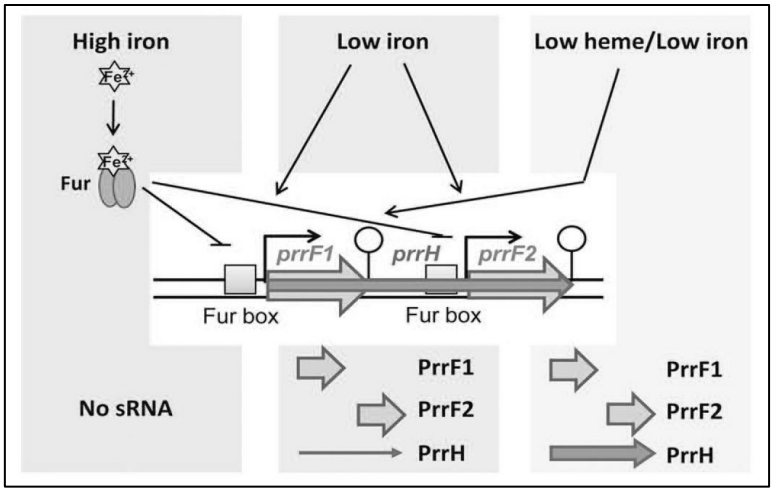

Fig.14 Genomic organization of genes involved in iron metabolism. During iron replete conditions Fur^{Fe2+} blocks *prrF1/2* transcription, if iron is scarce *prrF1/2* are transcribed and through base-pairing to their target mRNAs prevent translation. The sRNA PrrH is thought to act in a similar way.
⊥, negative control; ↓, positive control; (modified from Sonnleitner *et al.,* 2012).

In *P.aeruginosa* two almost identical sRNAs PrrF1/2 were shown to be activated during iron limitation when Fur does not function as a repressor. The sRNAs are encoded in tandem and each is flanked by one Fur box (Fig.14). Both sRNAs are functional orthologous of *E. coli* RhyB and act by base-pairing with target mRNAs, which encode genes involved in iron storage and required for protection from oxidative stress (Wilderman *et al.,* 2004). However, the

majority of affected genes encode enzymes that participate in aerobic and anaerobic metabolism (Vasil, 2007). Moreover, Oglebsky *et al.* (2008) reported that PrrF represses genes required for anthranilate degradation, which is a precursor of the PQS (*Pseudomonas* quinolone quorum sensing signal). Although Prrf1/2 are proposed to belong to the group of base-pairing sRNAs, up to now no direct interaction with potential target mRNAs has been experimentally verified.

Recently a third sRNA PrrH located between *prrF1/2* was discovered (Fig.14). It is induced by heme-regulated antitermination of *prrF1* transcription but the exact mechanism remains unknown (Oglesby-Sherrouse *et al.,* 2010). A model put forward by Oglesby-Sherrouse *et al.* (2008) proposed that PrrF1/2 and PrrH act in concert to regulate oxidative stress protection, iron storage and the production of iron containing enzymes.

The sRNA PhrS

PhrS is a 213 nucleotides long sRNA, which is expressed during stationary phase and requires the anaerobic regulator ANR for transcription. Under oxygen limiting conditions ANR interacts with a conserved ANR box in the *phrS* promoter region which leads to transcription of *phrS* (Sonnleitner *et al.*, 2011). Hfq has an indirect positive effect on *phrS* transcription because it is required for ANR expression (Sonnleitner *et al.*, 2011).

A transcriptome analyses performed by Sonnleitner *et al.* (2011) showed that in a PAO1Δ*phrS* strain genes required for synthesis of Pseudomonas quinolone signal (PQS) and for pyocyanin (PYO) were down-regulated. Indeed, strains overexpressing *phrS* showed increased levels of PQS and PYO. All genes which were influenced by the absence of PhrS are positively controlled by the transcriptional regulator PqsR. This suggested that PhrS regulates the *pqsR* mRNA. The *pqsR* gene is co-transcribed with a short upstream open reading frame (*uof*) (Fig.15, top left corner). Structural mapping showed that the *uof-pqsR* mRNA forms a secondary structure which masks both, the *uof* and *pqsR* ribosome binding sites. It could be shown that PhrS binds to the leader sequence of the *uof-pqsR* mRNA, which results in changes of the secondary structure of the mRNA and permits translation of the *uof* and of *pqsR* as translation of the latter was shown to be positively coupled to that of *uof*. (Sonnleitner *et al*., 2011).

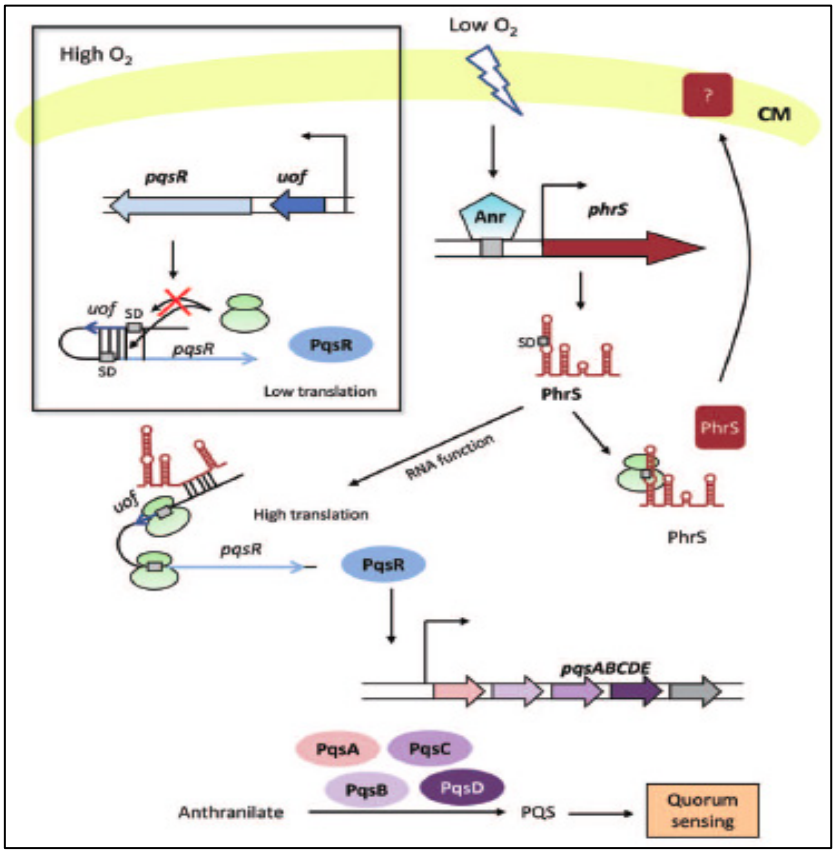

Fig.15 Model of PhrS action. At high oxygen levels translation of the quorum sensing regulator *pqsR* and an upstream open reading frame (*uof*) is blocked by a secondary structure sequestering the Shine and Dalgarno sequences. Upon oxygen limitation the oxygen responsive regulator ANR activates transcription of the sRNA *phrS*. Base-pairing of PhrS to a sequence 5' of the *uof* disrupts the secondary structure and enables *uof* as well as *pqsR* translation. In turn PqsR regulates the PQS biosynthesis operon and thereby influences quorum sensing. Additionally PhrS encodes a short protein with unknown function. (Sonnleitner *et al.,* 2011)

The sRNA PhrS links oxygen availability and quorum sensing which regulates virulence factor production. Under low O_2 conditions ANR activates PhrS synthesis which in turn enables translation of *pqsR*, which is the regulator of genes involved in biosynthesis of the quorum sensing signaling molecule PQS (Sonnleitner *et al.,* 2011) (Fig.15, right). Interestingly, PhrS also encodes for a highly conserved 37 amino-acid polypeptide whose function is as yet unknown (Sonnleitner *et al*., 2008).

The sRNA RgsA

The sRNA RgsA (regulation by GacA and stress) is mainly synthesized during stress and in stationary phase. It is dependent on the sigma factor RpoS. GacA most likely induces RgsA in an indirect manner. RgsA was shown to bind to the Hfq protein, which might suggest that RgsA acts by base-pairing with mRNAs (Sonnleitner *et al.*, 2012). Further, preliminary experiments showed that deletion of *rgsA* renders the mutant less resistant towards exposure to H_2O_2. This may indicate that genes required for protection against oxidative stress are potential targets of RgsA (González *et al.,* 2008).

1.4 PaeII and PaeIII, two newly described sRNAs in *P. aeruginosa*

The present study will focus on two sRNAs which were reported by Sorger-Domenigg (2010). A bioinformatic screen using an algorithm described by Lenz *et al.* (2004) was performed.
A potential new sRNA had to fulfill four premises:

- Sequence length between 50 and 300 nucleotides
- Located in intergenic regions
- The upstream region of a potential candidate must contain a σ^{54} or a σ^{70} binding site
- The presence of a rho-independent terminator

The screen predicted two new sRNAs termed PaeII and PaeIII. Using Northern blot analyses Sorger-Domenigg (2010) could verify the expression of both small RNAs.

1.4.1 The sRNA PaeII

The sRNA PaeII is encoded between the open reading frames PA4040 and PA4041 (Stover *et al.,* 2000), which are not characterized. Full length PaeII is 108 nucleotides in length and predicted to contain two stem-loop structures and features a rho-independent terminator (Fig.16). Cleavage by RNase E (cleavage site is indicated by an arrow in Fig.16) results in a 70nt processed variant, which is the abundant form of PaeII in the cell.

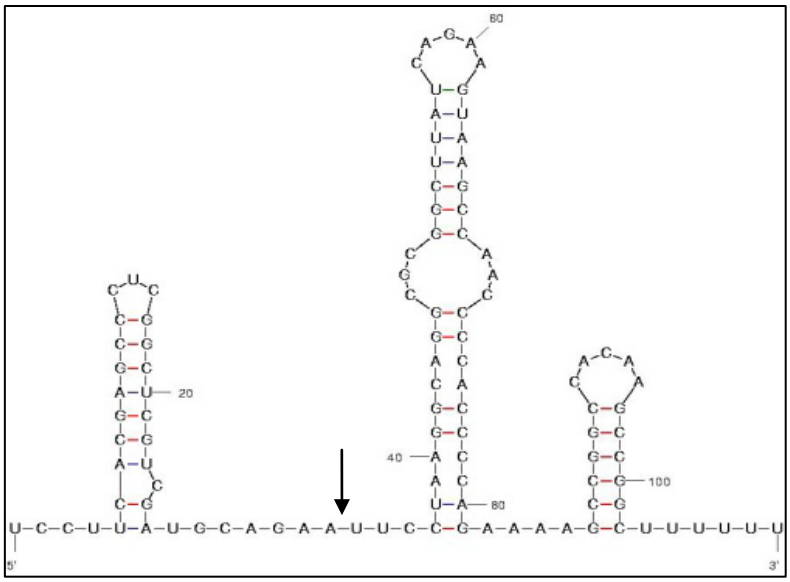

Fig.16 Putative secondary structure of PaeII. Secondary structure was predicted using the webserver mfold (Zuker, 2003). The arrow indicates the RNase E processing site (modified from Sorger-Domenigg, 2010).

Small RNAs are known to act in response to stress conditions and as regulators of different metabolic processes. This applies to PaeII, which is mainly transcribed during nutrient limitation. Upon starvation *P. aeruginosa* synthesizes the alarmone ppGpp which is needed for σ^{54}- (RpoN) dependent transcription. Although the *paeII* gene is also preceded by a putative σ^{70}-dependent promoter, *paeII* was shown to be transcribed at high ppGpp levels in a RpoN- dependent manner. PaeII transcription can be detected throughout growth being highest at the beginning of stationary phase.

Since a PAO1*hfq*$^{-}$ strain showed decreased levels of PaeII, it was tested if the RNA chaperone also interacts with PaeII. Hfq binds to the sRNA, but it did not have an impact on the stability of PaeII. Most likely Hfq has an indirect effect on *paeII* transcription.

Proteome and transcriptome analyses were performed to elucidate the physiological role of PaeII in minimal medium supplemented with mannitol (20mM) (Sorger-Domenigg, 2010).

Upon expression of *paeII* the proteins encoded by PA1244 and *mvaT* showed a higher abundance whereas proteins encoded by PA1579 and PA3332 where induced in the absence of PaeII. MvaT is a transcriptional regulator involved in arginine metabolism, prophage activation and pyocyanin synthesis. However a pyocyanin assay showed no differences between PAO1 and PAO1Δ*paeII* with regard to pyocyanin production.

A microarray assay revealed a surprisingly low number of genes, which were significantly affected by PaeII when grown in BM2 medium supplemented with mannitol (20mM).

Upon PaeII expression two genes were up- and seven were down-regulated. Among potential PaeII targets was an operon formed by genes PA3515-PA3519, which encodes for proteins of unknown function (PA3515, PA3518 and PA3519) as well as for hypothetical lyases (PA3516 and PA3517). The most down-regulated gene *sdsA1* encodes the zinc dependent SDS hydrolase SdsA1 (Sorger-Domenigg, 2010).

1.4.2 The sRNA PaeIII

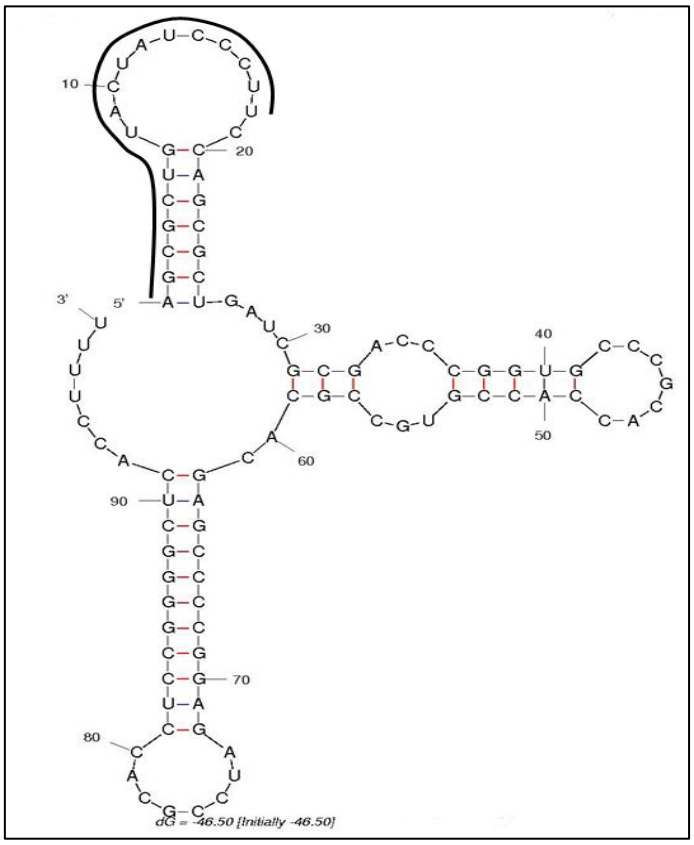

Fig.17 Putative secondary structure of PaeIII. Secondary structure was predicted using the webserver mfold (Zuker, 2003). The predicted interaction site of PaeIII with the *rpoS* mRNA is indicated by a black line (modified from Sorger-Domenigg, 2010).

PaeIII is 98 nucleotides in length and encoded between the open reading frames PA3535, encoding a probable serine protease, and PA3536, encoding a hypothetical protein (Stover *et al.,* 2000).The sRNA is transcribed from a σ^{70}- dependent promoter and is not processed. According to secondary structure predictions, PaeIII consists of two-stem loops and contains a rho-independent terminator (Fig.17). PaeIII expression is dependent on the growth phase and RsmA. PaeIII levels are increased during stationary growth. Moreover, growth in

synthetic sputum cystic fibrosis medium and exposure to human serum increased *paeIII* transcription, which suggested that PaeIII might play a role during infection. The sRNA was shown to bind to Hfq with low affinity (Kd~100nM), and its stability was not affected by the presence or absence of Hfq.

Proteome and transcriptome analyses were performed. A microarray experiment performed by Sorger-Domenigg (2010) revealed potential targets of PaeIII. Taken together, the transcriptome analysis showed that genes involved in type three secretion system (T3SS) and antibiotic resistance were up-regulated, and that genes required for anaerobic respiration and pyocin production were repressed, in the presence of PaeIII.

Furthermore, the *rpoS* gene, encoding the stationary phase sigma factor, was identified as a potential target mRNA. The mRNA levels of *rpoS* were 2.9 fold down-regulated upon PaeIII expression. Bioinformatic analyses using the software TargetRNA (Tjaden *et al.,* 2006) predicted a possible interaction between the first loop of PaeIII and the Shine and Dalgarno sequence of *rpoS* (Fig.18). Upon *paeIII* expression a decrease in translation of a *rpoS::lacZ* reporter gene was observed (Sorger-Domenigg, 2010).

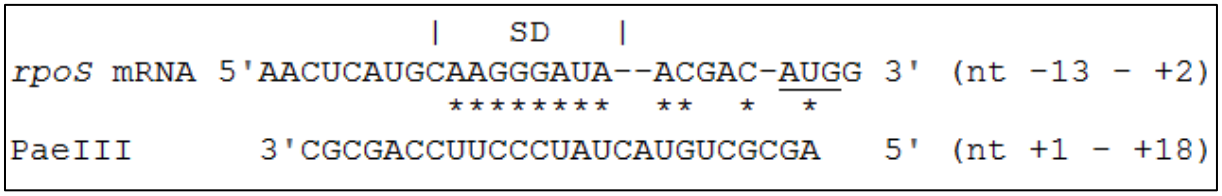

Fig.18 The possible interaction site of PaeIII with the Shine and Dalgarno sequence of *rpoS* mRNA. The complementary region of PaeIII and the *rpoS* mRNA is shown. Possible base-pair interactions are denoted by stars. The Shine and Dalgarno sequence (SD) of *rpoS* mRNA is indicated.

1.5 Aims of the study

Two novel *Pseudomonas aeruginosa* sRNAs were biocomputationally identified and experimentally verified by Sorger-Domenigg (2010). They were termed PaeII and PaeIII. Proteome and transcriptome analyses were performed to reveal the potential roles of both sRNAs in *Pseudomonas* strain PAO1. This study primarily aimed at providing a more in depth analyses on the role of PaeII by employing high throughput sequence analyses. In addition, it was attempted to verify the possible regulatory role of PaeIII on *rpoS* expression.

2. Results

2.1 *PaeIII* transcription is independent of RpoS and ANR

The software PATSER (http://liv.bmc.uu.se:16080/rsa-tools, Pattern Matching, PATSER) was designed to find locations of patterns in genomic sequences. Sorger-Domenigg (2010) used this software to screen the *P. aeruginosa* intergenic regions for potential σ^{54} or σ^{70}-binding sites. The obtained results were combined with other bioinformatic analyses, which enabled Sorger-Domenigg (2010) to detect two potential new *P. aeruginosa* sRNA candidates. One of those candidates is the sRNA PaeIII, which was predicted to be transcribed in a σ^{70}-dependent manner (Fig.19) (Sorger-Domenigg, 2010).

```
          -35 TTGACA            -10 TAATAT        →
5' AACTAAAGGATTCATGCGAAATGCATAGCGCTATACTGTCCATCCAGCGCTGTACTATCCCTTCC
AGCGCTGATCGCGACCCGGTGCCCGCACCACCGTGCCGCACGAGCCCCGGAGATCCGCACCTCCGGGG
CTCACCTTTTGCTCCTCTCGCA 3'
```

Fig.19 σ^{70} promoter region of *paeIII*. The σ^{70} -35 and -10 promoter elements are shown in bold and the consensus binding motif is shown above. The arrow indicates the *paeIII* transcriptional start site. The full sequence of *paeIII* is underlined.

The sRNA PaeIII is mainly expressed during stationary phase and, apart from a potential σ^{70}-binding site, it displays putative RpoS and ANR binding sites (Fig.20). ANR is an oxygen-responsive regulator, which is only active under anaerobic conditions which prevail during stationary phase (Zimmermann *et al.,* 1991). The TTGATN$_4$GTCAA consensus motif for ANR is located ~40nt upstream of the transcriptional start.

The alternative σ factor RpoS of *P.aeruginosa*, which is preferentially synthesized during stationary phase, controls ~14% of all genes during the late growth phase (Fujita *et al.,* 1994; Schuster *et al.,* 2004). The RpoS binding motif CTATACT is found 10 nts upstream of the transcriptional start site (Winteler and Haas, 1996; Schuster *et al.,* 2003) (Fig. 20).

```
                TTGAT-----ATCAA      CTATACT          →              ********
5' AACTAAAGGATTCATGCGAAATGCATAGCGCTATACTGTCCATCCAGCGCTGTACTATCCCTTCC
AGCGCTGATCGCGACCCGGTGCCCGCACCACCGTGCCGCACGAGCCCCGGAGATCCGCACCTCCGGGG
CTCACCTTTTGCTCCTCTCGCA 3'
```

Fig.20 The genomic location of *paeIII*. The arrow indicates the *paeIII* transcriptional start site. The sequence of *paeIII* is underlined. The predicted interaction site of PaeIII with *rpoS* mRNA is indicated with a box. The stars denote the deleted region in the PaeIII variant PaeIII$_{\Delta 10\text{-}19}$. The putative ANR box is indicated in bold and italicized letters and the consensus binding motif is shown above. The putative RpoS recognition motif is shown in bold and underlined. The consensus RpoS binding motif is shown above.

To investigate the influence of the predicted RpoS and ANR binding motifs on *paeIII* transcription, the strains PAO1, PAO1*rpoS*$^-$ and PAO1*anr*$^-$, were grown in LB-medium and samples were removed when the cultures reached an OD_{600} of 2.2 and 3.5, respectively. The optical densities represent exponential and stationary growth phase. After total RNA extraction, a Northern-blot analysis (Fig. 21) was performed using a primer specific for *paeIII* (W28). The 5s rRNA served as a control and was detected with primer I26 .

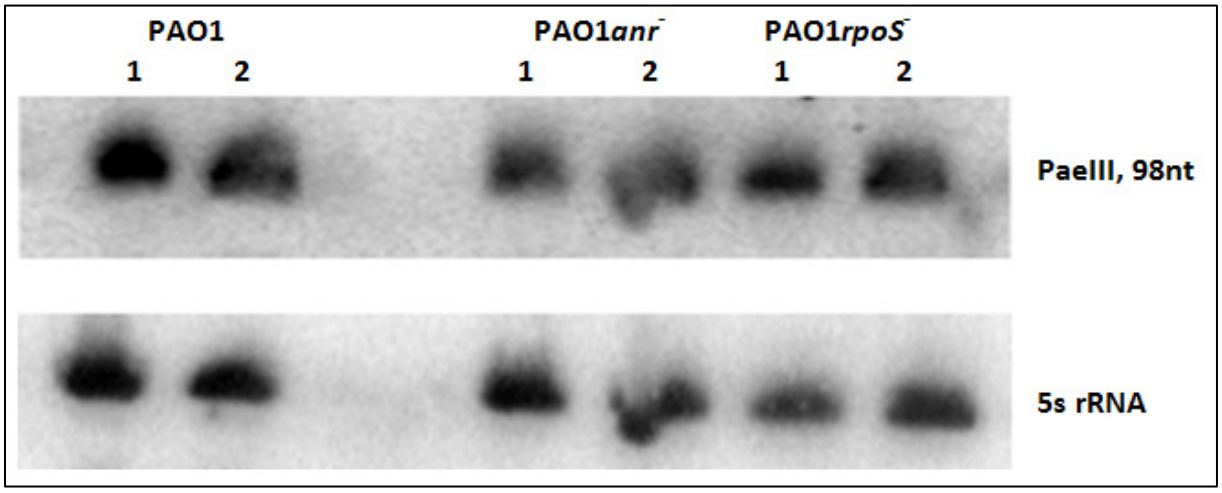

Fig.21 *PaeIII* transcription does not depend on RpoS and ANR. The strains PAO1, PAO1*anr*$^-$ and PAO1*rpoS*$^-$ were grown in 20ml LB medium. When the cultures reached an OD_{600} of 2.2 (Lanes 1) and 3.5 (Lanes 2), respectively, samples for extraction of total RNA were withdrawn. 5µg of total RNA was used for the Northern-blot. The oligonucleotide W28 was used for detection of the sRNA PaeIII. The 5s rRNA served as loading control and was detected using the oligonucleotide I26.

The Northern-blot analysis revealed no differences between PaeIII levels in the wild type PAO1 strain when compared with the two mutant strains (Fig.21). Therefore, under the conditions tested, transcription of *paeIII* appears to be neither dependent on RpoS nor on ANR.

2.2 Effect of PaeIII on *rpoS* translation

A microarray analysis performed by Sorger-Domenigg (2010) compared the transcriptome profiles of a PaeIII sRNA expressing strain and a strain harboring a deletion of *paeIII*. The abundance of *rpoS* mRNA was reduced in the presence of PaeIII. For validation of a potential target of a sRNA, *lacZ* fusions represent a robust and well established reporter-gene system. Possible gene regulation caused by the sRNA can be monitored by comparing the β-galactosidase activity conferred by the reporter-gene in cells, which express the sRNA with that of mutant strains deleted for the sRNA (Vogel, 2007). Sorger-Domenigg (2010) observed that the translation of *rpoS::lacZ* mRNA was decreased in the presence of PaeIII. A bioinformatic analysis revealed a possible PaeIII-*rpoS* mRNA binding site that covers the Shine and Dalgarno sequence of *rpoS* (Sorger-Domenigg, 2010) (Fig.18).

To verify the PaeIII-*rpoS* interaction a *paeIII* overexpressing plasmid was constructed, wherein the *paeIII* sequence, comprising a part of the putative interaction site of PaeIII with *rpoS* mRNA, was deleted (see Fig.20). The plasmid was termed pJTPaeIII$_{\Delta 11\text{-}18}$. A translational *rpoS::lacZ* fusion, containing the *rpoS* promoter and the first 56 codons (pME6014*rpoS*) was used to monitor possible effects of the sRNA on *rpoS* translation.

The strains PAO1Δ*paeIII*(pJT19, pME6014*rpoS*), PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*) and PAO1Δ*paeIII*(pJTPaeIII$_{\Delta 11\text{-}18,}$ pME6014*rpoS*) were grown in triplicates in LB-medium (Fig.23). When the cultures reached an OD_{600} of 1.0, the synthesis of PaeIII and the PaeIII$_{\Delta nt11\text{-}18}$ variant, respectively, was induced by addition of toluate (2mM). Then, samples for isolation of total RNA and for determination of the β-galactosidase activitiy conferred by the *rpoS::lacZ* mRNA were withdrawn every hour.

First, a Northern-blot analysis was performed to verify PaeIII and PaeIII$_{\Delta 11\text{-}18}$ expression. As expected, no PaeIII sRNA was detected in strain PAOΔ*paeIII*(pJT19, pME6014*rpoS*) (Fig.22). In contrast, expression of PaeIII and of the PaeIII variant PaeIII$_{\Delta 11\text{-}18}$ could be verified in strain PAOΔ*paeIII*(pJTPaeIII, pME6014*rpoS*) and in strain PAOΔ*paeIII*(pJTPaeIII$_{\Delta 11\text{-}18,}$ pME6014*rpoS*), respectively (Fig.22).

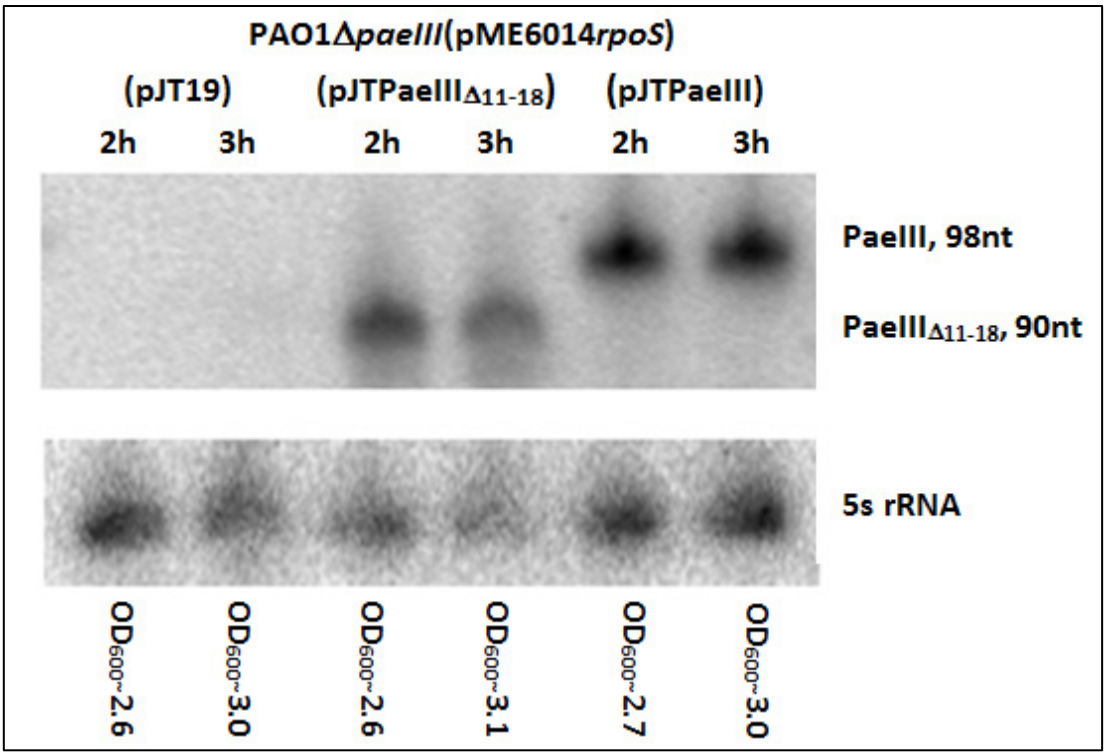

Fig.22 Verification of sRNA expression from plasmids. The strains PAO1Δ*paeIII*(pJT19, pME6014*rpoS*), PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*) and PAO1Δ*paeIII*(pJTPaeIII$_{\Delta 11-18}$, pME6014*rpoS*) were grown in 20ml LB medium. Samples were taken 2 and 3 hours post induction of PaeIII and PaeIII$_{\Delta 11-18}$, synthesis. The corresponding optical densities are indicated. After RNA extraction, 5µg of total RNA was used for the Northern-blot analysis. The oligonucleotide W28 was used for detection of PaeIII. The 5s rRNA served as a loading control and was dected using the oligonucleotide I26.

A comparison between the correspondent β-galactosidase activities conferred by plasmid pME6014*rpoS* in strains PAO1Δ*paeIII*(pJT19, pME6014*rpoS*) and PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*) indicated, that PaeIII repressed *rpoS::lacZ* translation (Fig.23). In contrast, the mutant form of PaeIII, PaeIII$_{\Delta 11-18}$, failed to repress *rpoS::lacZ* translation. These results indicated that the sRNA PaeIII represses *rpoS* translation (Fig.23).

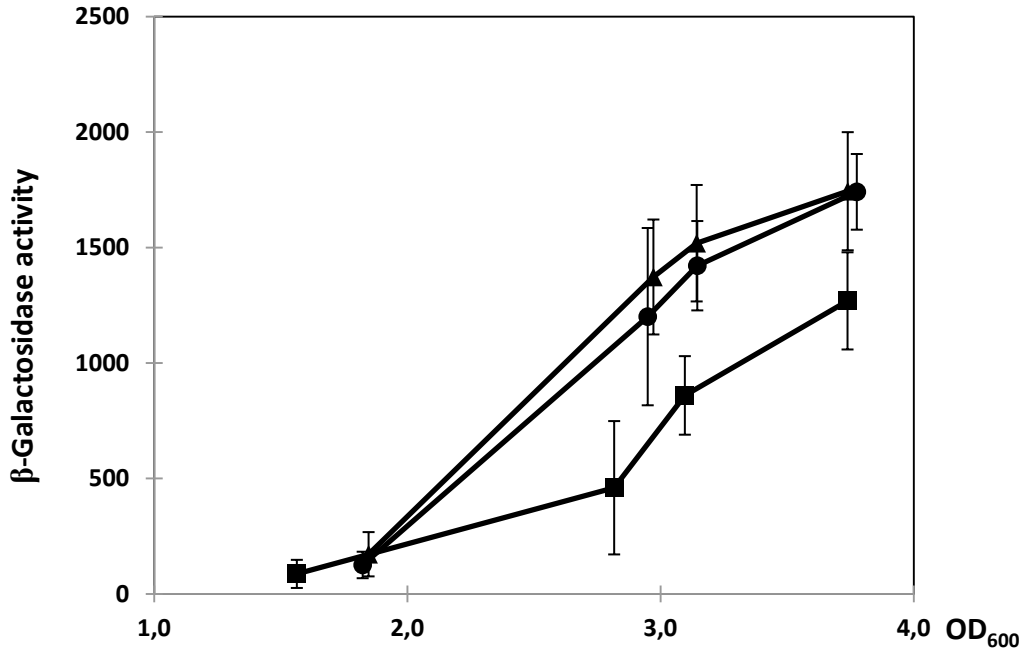

Fig.23 *RpoS* translation is repressed by PaeIII. The strains PAO1Δ*paeIII*(pJT19, pME6014*rpoS*)(●), PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*)(■) and PAO1Δ*paeIII*(pJTPaeIII$_{\Delta 11\text{-}18}$, pME6014*rpoS*)(▲) were grown in triplicates in 20ml LB medium. At an OD_{600} of 1.0 the PaeII synthesis was induced by addition of toluate (2mM). Samples for measurement of β-galactosidase activities and for total RNA isolation were withdrawn every hour. The β-galactosidase activities conferred by the translational *rpoS::lacZ* fusion (pME6014*rpoS*) were determined as a function of growth. The mutant PaeIII$_{\Delta 11\text{-}18}$ sRNA showed a reduced repression of *rpoS::lacZ* translation. The values shown were derived from two independent experiments. The error bars represent standard deviations.

2.3 Compensatory mutations in PaeIII and *rpoS*

To verify the putative interaction of PaeIII with *rpoS* mRNA (Fig.18) and to determine if base-pairing at the proposed binding site is required for regulation, the DNA sequences of both, *paeIII* and *rpoS,* were modified to carry compensatory mutations in the predicted interaction site (Fig.24A). By site directed mutagenesis the nucleotides -1, -4 and -5 with regard to the A (+1) of the start codon of *rpoS::lacZ* mRNA were changed from A**AC**GA**C**AUG to A**GT**GA**T**AUG (*rpoS*mut::*lacZ*, Fig.24A). The corresponding complementary nucleotides +4, +7 and +8 with regard to the A (+1) of the transcriptional start site of PaeIII were changed from A**UG**UC**G**CGA to A**CA**UC**A**CGA (PaeIIImut, Fig.24A). The mutated sRNA PaeIIImut was transcribed from plasmid pJTPaeIIImut and the mutated *rpoS*mut::*lacZ* fusion gene was harboured by plasmid pME6014*rpoS*mut.

We hypothesized that after introduction of mutations within the predicted interaction site of *paeIII*, the sRNA would be unable to repress *rpoS::lacZ* translation. In contrast, the introduction of compensatory mutations in *rpoS*mut*::lacZ* was anticipated to restore inhibition of translation in presence of PaeIIImut. To test this, the strains PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*) and PAO1Δ*paeIII*(pJTPaeIIImut, pME6014*rpoS*), respectively, were grown in LB-medium and the β-galactosidase activities conferred by the *rpoS::lacZ* fusion were determined during growth (Fig.24B). In a second experiment, the strains PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*mut) and PAO1Δ*paeIII*(pJTPaeIIImut, pME6014*rpoS*mut) were grown in LB-medium and the β-galactosidase activities conferred by the *rpoS*mut*::lacZ* fusion were measured during growth (Fig.24C).

As shown in Fig.24B, upon introduction of three point mutations in PaeIIImut, repression of *rpoS::lacZ* translation by PaeIIImut was hardly affected. The β-galactosidase levels conferred by the *rpoS*mut*::lacZ* fusion (Fig.24C) were decreased when compared to those conferred by the *rpoS::lacZ* fusion (Fig.24B). This might be due to the mutations close to the Shine and Dalgarno sequence of *rpoS*mut::lacZ (Fig.24A), which may interfere with translation and/or mRNA stability. Furthermore, *rpoS*mut*::lacZ* translation was undistinguishable in the presence of PaeIII or PaeIIImut (Fig.24C). Taken together, these results (24B/C) suggested that introduction of three point muations within the predicted interaction site, neither in PaeIII nor in *rpoS::lacZ,* was sufficient to reduce PaeIII mediated translational repression.

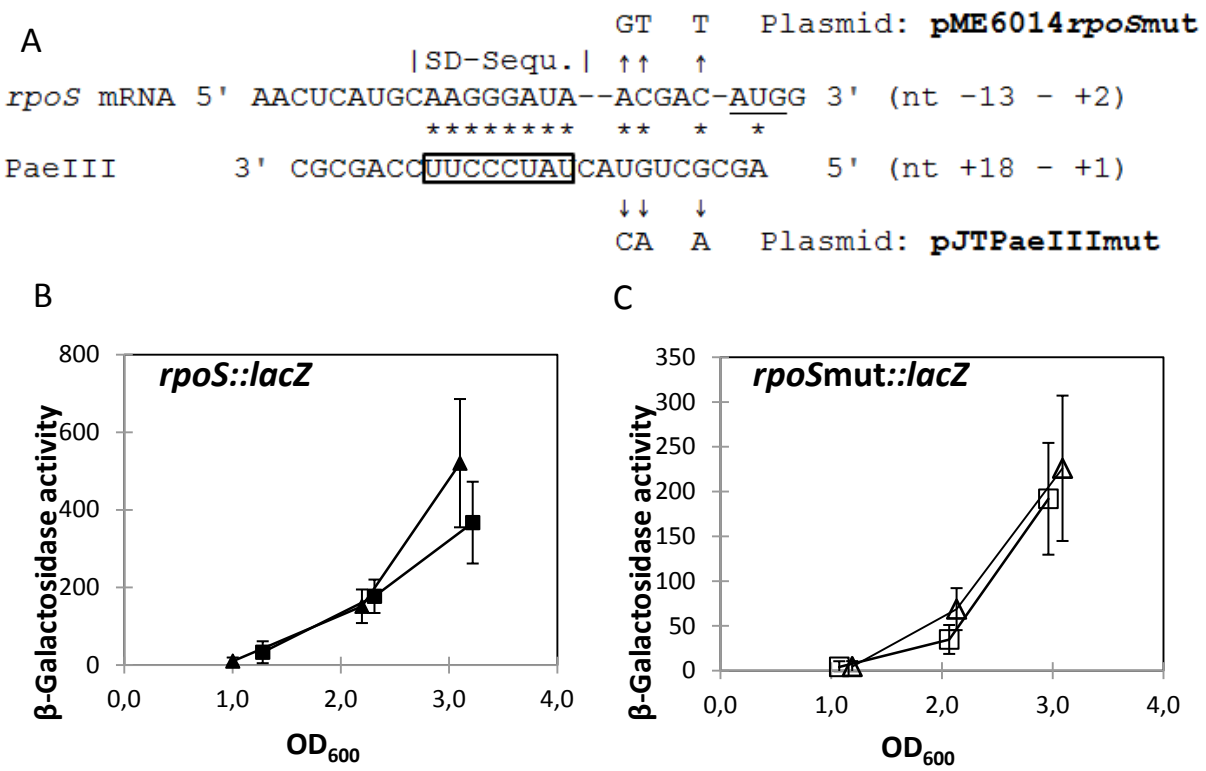

Fig.24 Compensatory mutations.

A. The putative PaeIII sRNA and *rpoS* mRNA interaction site. The stars indicate complementarity between PaeIII and *rpoS* mRNA. Arrows indicate specific nucleotide changes in *paeIII* and *rpoS* generated by site-directed mutagenesis. Plasmids carrying the mutated RNAs are quoted in bold. The region deleted in plasmid pJTPaeIII$_{\Delta 11\text{-}18}$ which was used in the experiment shown in Fig.22 is boxed.

B. Mutations in the PaeIII sRNA hardly effected *rpoS::lacZ* translation. The strains PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*) (■) and PAO1Δ*paeIII*(pJTPaeIIImut, pME6014*rpoS*) (▲), were grown in LB medium to an OD_{600} of 1.0. Then, *paeIII* expression was induced by addition of toluate (2mM). Samples were withdrawn every hour and the β-galactosidase activities conferred by the *rpoS::lacZ* fusion gene were determined as a function of growth. The values shown were derived from two independent experiments. Error bars represent standard deviations.

C. Mutations in *rpoS*mut*::lacZ* reduced translation and the wild type PaeIII sRNA inhibits *rpoS*mut*::lacZ translation*. The strains PAO1Δ*paeIII*(pJTPaeIII, pME6014*rpoS*mut*::lacZ*) (□) and PAO1Δ*paeIII*(pJTPaeIIImut, pME6014*rpoS*mut*::lacZ*) (△), were grown in LB medium to an OD_{600} of 1.0. Then sRNA expression was induced by addition of toluate (2mM). Samples were withdrawn every hour and the β-galactosidase activities conferred by the *rpoS*mut*::lacZ* fusion gene were determined as a function of growth. The values shown were derived from two independent experiments. Error bars represent standard deviations.

2.4 Potential targets of PaeII revealed by RNA-sequencing

Small RNAs control gene expression by interfering with mRNA translation and/or they affect target mRNA stability (Waters and Storz, 2009). In either case, the levels of the corresponding mRNA are reduced. The changes in transcript levels can be monitored using whole transcriptome analyses. In general, microarray assays allow for detection of changes in gene-expression of annotated genes. In contrast, high throughput RNA-sequencing offers the possibility to monitor changes in transcription of protein-coding genes and intergenic regions. Therefore, this method can provide an in depth view on transcriptional changes at the scale of the entire genome under given growth conditions (Vliet, 2009; Wurtzel *et al.,* 2012).

Using a microarray analysis Sorger-Domenigg (2010) compared the transcription profile of the strain PAO1 with that of the *paeII* deficient strain PAO1Δ*paeII*, when grown in BM2 minimal medium supplemented with mannitol (20mM). Only a rather low number of genes were shown to be affected by PaeII expression (see table in appendix).

In the present study the experimental setup was modified and high throughput RNA sequencing was used to monitor the effect of PaeII overexpression on the transcriptome. Instead of using mannitol as a carbon source, in this study the BM2 minimal medium was supplemented with succinate (20mM). Furthermore, the sRNA PaeII was not expressed endogenously but expressed from plasmid pJTPaeII. The strains PAO1Δ*paeII*(pJT19) and PAO1Δ*paeII*(pJTPaeII) were grown in triplicates in 20 ml BM2- succinate medium to an OD_{600} of 1. Then, expression of PaeII was induced by addition of toluate (2mM) and after 30 minutes samples for RNA isolation were withdrawn. The growth rate of the *paeII* mutant strain was identical to the wild type-strain (data not shown). Successful overexpression of PaeII was confirmed by a Northern-blot analysis (Fig.25). The cDNA was synthesized and whole-trancriptome libraries for Illumina RNA-sequencing were prepared. After sequencing, the obtained reads were mapped onto the *Pseudomonas aeruginosa* PAO1 genome and a comparative gene expression analysis of the PaeII expressing and the *paeII* deletion strain was performed.

Two samples of each strain were used for the transcriptome analyses. Transcripts with p-values lower or equal to 0.05 were deemed to be differentially expressed (Fig.28). The

sequencing data are accessible and are represented in the software GenomeBrowser that can be accessed at http://genome.tbi.univie.ac.at/fgb2/gbrowse/J00007-PAO1/.

The GenomeBrowser provides the possibility to browse through the genome and depicts the number of reads for a given genome segment, which is proportional to the abundance of a given RNA. This tool was also used to confirm that PaeII is present in its processed form (Fig.26).

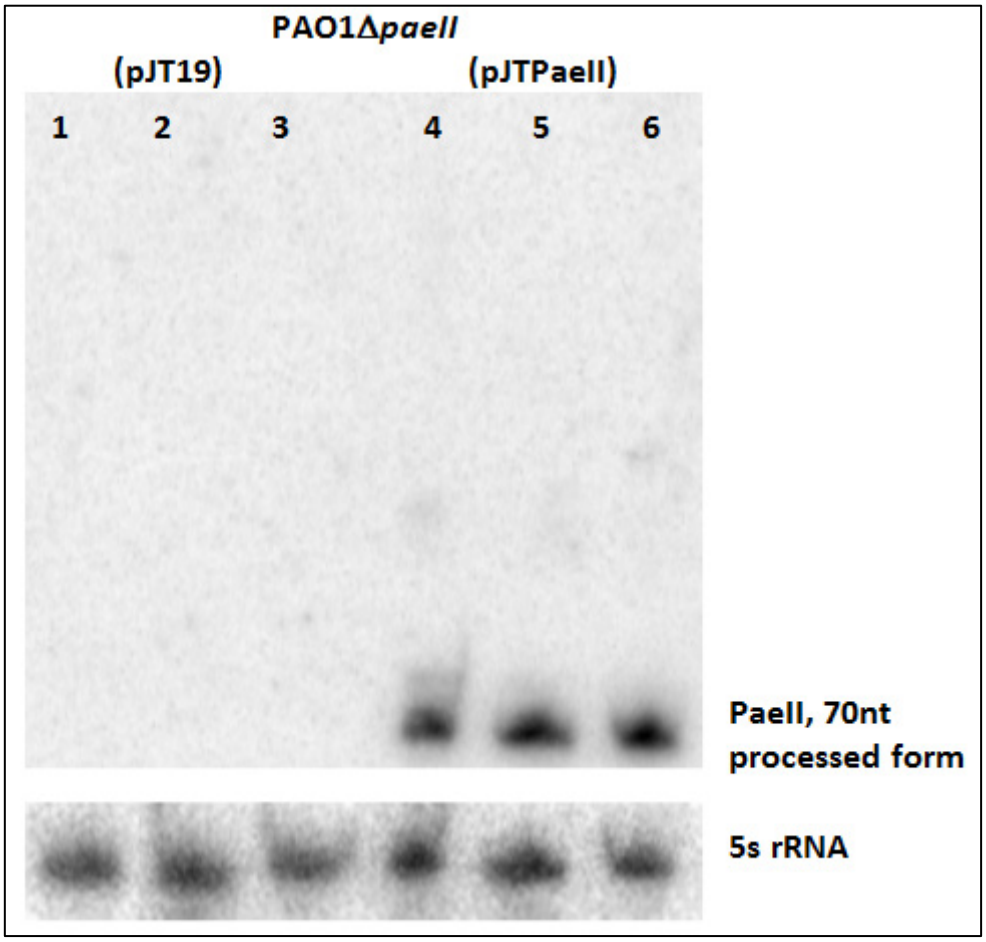

Fig.25 Verification of PaeII sRNA expression. The strains PAO1Δ*paeII*(pJT19) and PAO1Δ*paeII*(pJTPaeII) were grown in triplicates in BM2 medium supplemented with succinate (20mM). Upon reaching an OD of 1.0, the expression of PaeII was induced from plasmid pJTPaeII by addition of toluate (2mM). After 30 minutes samples for total RNA extraction were withdrawn. 5µg of total RNA was used for the Northern-blot analysis. The expression of the processed form of PaeII could be verified in all three replicates of strain PAO1Δ*paeII*(pJTPaeII). Lanes 1, 2, 3 represent RNA obtained from triplicate samples of strain PAO1Δ*paeII*(pJT19). Lanes 4, 5, 6 represent the RNA obtained from the triplicate samples of strain PAO1Δ*paeII*(pJTPaeII). For detection of the PaeII sRNA oligonucleotide V28 was used. The 5s rRNA served as loading control and was detected using oligonucleotide I26.

As expected, no PaeII was detected in strain PAO1Δ*paeII*(pJT19) and expression of PaeII could be confirmed in strain PAO1Δ*paeII*(pJTPaeII). When grown in BM2-succinate medium only the processed form of PaeII with a length of 70 nt was detected (Fig.25).

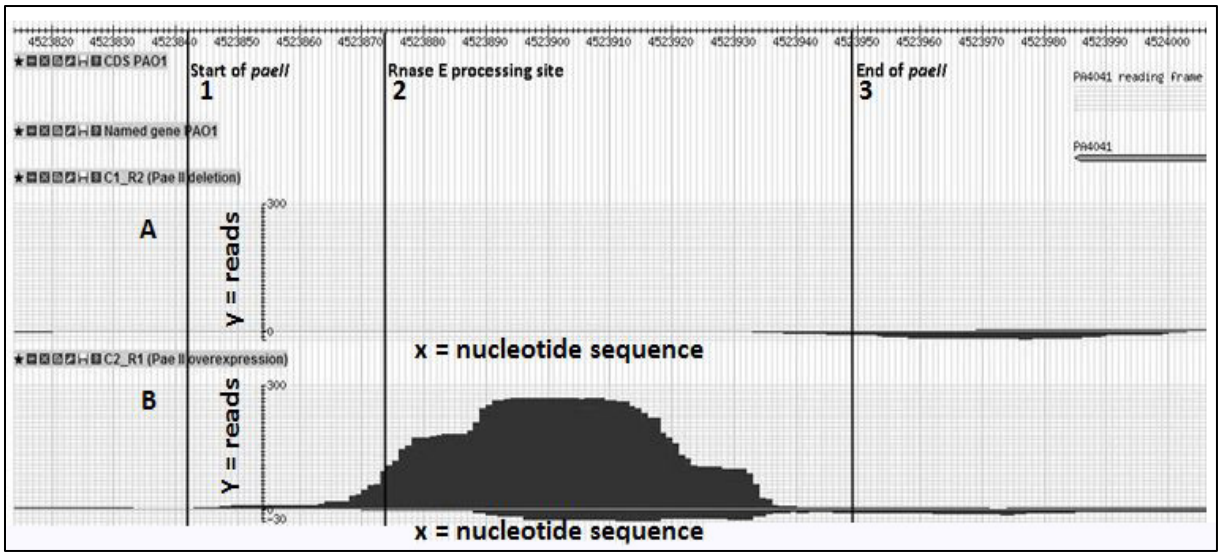

Fig.26 Only the processed form of PaeII is present in vivo. This picture is taken from the GenomeBrowser. Depicted is the intergenic region between genes PA4040 and PA4041. The x- axis represents the location in the genome and the y-axis shows the number of reads at a certain position. **A.** shows the sequencing result for the *paeII* deletion strain and **B.** shows the sequencing result for the PaeII expressing strain. The vertical line **1** represents the transcriptional start of *paeII*; the vertical line **2** represents the RNase E processing site of PaeII; the vertical line **3** represents the end of *paeII.*

Basically the same result was obtained by RNA-sequencing. In the PaeII expressing strain reads at the *paeII* genomic location were obtained (Fig.26, see region between bars 2 and 3). As obvious from the Northern-blot analysis (Fig.25), no reads for full length PaeII were observed (Fig.26, see region between bars 1 and 2).

A microarray analysis performed by Sorger-Domenigg (2010) compared the transcriptome profile of the strain PAO1 with that of an isogenic strain harbouring a deletion in the *paeII* gene, after growth in BM2 minimal medium supplemented with mannitol (20mM).

PaeII affected genes identified by Sorger-Domenigg (2010) were compared with the putative PaeII target mRNAs identified by the RNA-sequencing analysis. This comparison revealed that PaeII affected a different set of genes when grown in BM2-succinate medium.

In total 50 mRNAs were found to be differentially abundant after PaeII expression. When compared with strain PAO1Δ*paeII*(pJTPae19), 44 were up- and 6 were down-regulated in

strain PAO1Δ*paeII*(pJTPaeII). PaeII overexpression affected genes coding for (i) membrane proteins, (ii) for proteins involved in transport of molecules and (iii) genes which are part of transmembrane transporters (Figure 27).

According to the pseudomonas genome database (www.pseudomonas.com), among the 19 unknown genes, 11 are hypothetically involved in the afore mentioned processes (Fig.27, *).

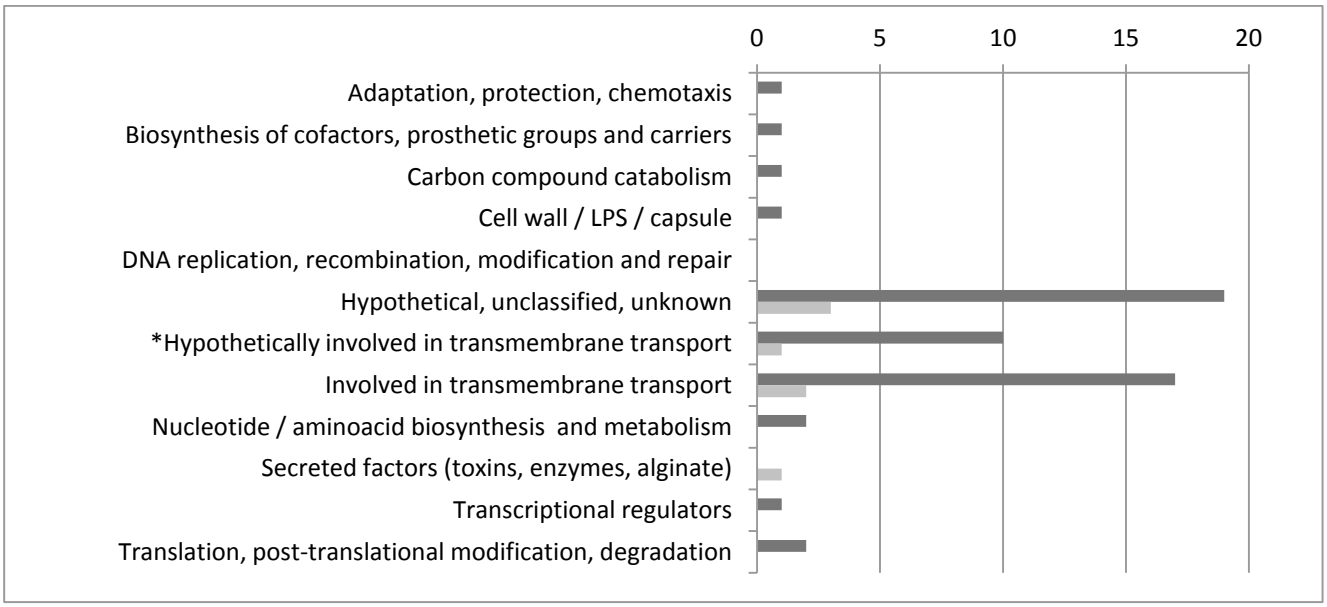

Fig.27 Functional classes of genes which are affected after expression of PaeII. The number of genes belonging to different functional classes, that are up- (black) or down- (gray) regulated after *paeII* expression are shown.

Down-regulated transcripts sorted by PA gene numbers:

[a]Verified manually	Gene description	Id/Gene name		[b]Fold change	Pval	Strand
~	Transport of small molecules	PA0291	*oprE*	0,64	3,72E-02	-
~	Hypothetical, unclassified, unknown		PA0716	0,55	3,44E-02	+
~	Transport of small molecules	PA0958	*oprD*	0,64	5,11E-02	-
-	Secreted Factors (toxins, enzymes, alginate)	PA0985	*pyoS5*	0,59	3,13E-02	+
~	Hypothetical, unclassified, unknown		PA1371	0,45	3,18E-03	+
~	Secreted Factors (toxins, enzymes, alginate)	PA1900	*phzB2*	0,60	1,49E-02	+
~	Secretion system		PA2367	0,53	1,94E-02	-
-	Hypothetical, unclassified, unknown		PA2372	0,50	3,07E-02	+
-	Biosynthesis of cofactors, prosthetic groups and carriers, Amino acid biosynthesis and metabolism	PA4695	*ilvH*	0,53	1,01E-02	-
-	Biosynthesis of cofactors, prosthetic groups and carriers, Amino acid biosynthesis and metabolism	PA4696	*ilvI*	0,61	3,35E-02	-

Up-regulated transcripts sorted by PA gene numbers:

[a]Verified manually	Gene description	Id/Gene name		[b]Fold change	Pval	Strand	
+	Trans membrane transporter		PA0781	10,15	2,90E-21	-	
+	Carbon compound catabolism	PA0887	*acsA*	2,18	1,42E-02	+	
+	Export signal		PA1324	1,54	4,37E-02	+	
~	Nad transport / membrane		PA1559	1,76	4,22E-02	+	
+	Export signal		PA1592	1,63	2,22E-02	-	
-	Nucleotide biosynthesis and metabolism	PA1920	*nrdD*	2,09	3,64E-02	-	
-	Biotin biosynthesis		PA1921	8,87	6,51E-08	-	
~	Probable TonB-dependent receptor	PA1922	*cirA*	17,02	5,68E-25	+	
~	Biosynthesis of cofactors, prosthetic groups and carriers		PA1923	11,67	6,44E-22	+	
~	Biopolymer transport		PA1924	8,57	1,01E-07	+	
+	Hypothetical, unclassified, unknown		PA1925	10,77	4,83E-13	+	
~	Amino acid biosynthesis and metabolism	PA1927	*metE*	2,55	4,06E-05	+	
-	Type 1 export signal		PA2434	2,44	9,46E-03	-	
-	Transport of small molecules		PA2435	1,88	2,11E-02	-	
~	Membrane protease		PA2438	2,10	4,24E-02	-	
~	Membrane proteins		PA2439	2,04	1,66E-02	-	
~	Biotin biosynthesis		PA2679	1,99	1,49E-02	-	
-	Probable TonB-dependent receptor		PA2911	2,75	4,95E-04	+	
~	Probable ATP-binding component of ABC transporter		PA2912	2,39	4,47E-03	+	
~	Transport of small molecules		PA2913	2,18	5,04E-03	+	
~	Membrane proteins, transport of small molecules		PA2914	3,01	3,29E-04	+	
+	Probable porin		PA3038	1,62	2,52E-02	+	
-	Signal transduction		PA3233	1,84	2,97E-02	-	
+	Membrane proteins, transport of small molecules		PA3234	2,49	1,45E-04	-	
+	Membrane proteins		PA3235	2,25	9,64E-04	-	
-	DNA replication, recombination, modification and repair		PA3596	2,58	1,29E-02	-	
-	Membrane proteins, transport of small molecules		PA3597	2,30	1,42E-02	-	
~	Amidase activity		PA3598	5,48	5,08E-08	-	
~	Translation, post-translational modification, degradation		PA3600	9,59	5,06E-06	-	Zur
~	Translation, post-translational modification, degradation		PA3601	8,14	5,91E-20	-	Zur
+	Membrane proteins		PA3819	1,58	3,43E-02	-	
~	Type 1 export signal		PA4063	4,70	7,76E-06	+	Zur
+	Probable ATP-binding component of ABC transporter		PA4064	4,45	9,07E-05	+	
+	Membrane proteins		PA4065	4,32	6,81E-06	+	
+	Type 1 export signal		PA4066	2,94	6,18E-04	+	
~	Oxidation reduction process transmembrane		PA4170	3,59	2,64E-06	+	

[a]Verified manually	Gene description	Id/Gene name		[b]Fold change	Pval	Strand	
~	Adaptation, protection, chemotaxis		PA4290	2,12	2,34E-02	-	
+	Hypothetical, unclassified, unknown		PA4782	1,88	1,20E-02	-	
+	Membrane proteins		PA4834	9,60	4,24E-18	-	
~	Membrane proteins		PA4835	12,86	6,04E-11	-	
~	Hypothetical, unclassified, unknown		PA4836	13,52	6,89E-18	-	
+	Probable OM protein precursor		PA4837	14,28	2,20E-27	-	
-	Membrane protein		PA4838	5,39	5,98E-10	+	Zur
+	Osmotically inducible lipoprotein OsmE	PA4876	*osmE*	1,84	8,84E-03	+	
~	Membrane proteins, transport of small molecules	PA5501	*znuB*	1,76	4,81E-02	+	
~	Biosynthesis of cofactors		PA5532	3,65	3,49E-05	-	
~	Hypothetical, unclassified, unknown		PA5534	7,36	4,05E-13	-	Zur
+	Biosynthesis of cofactors		PA5535	13,00	7,08E-16	-	Zur
+	Transcriptional regulator DksA2	PA5536	*dksA2*	12,29	1,96E-07	-	Zur
~	Type 1 export signal		PA5537	4,72	4,76E-07	+	
+	Cell wall / LPS / capsule	PA5538	*amiA*	17,29	8,91E-26	-	
+	Biosynthesis of cofactors		PA5539	14,71	1,57E-22	+	Zur
+	Phenylacetate catabolic process		PA5540	22,51	6,37E-20	+	Zur
+	Dihydrooratase	PA5541	*pyrQ*	33,12	1,67E-23	+	Zur

Table 2 Transcripts which show a different abundance in strain PAO1Δ*paeII* (pJTPaeII) when compared to strain PAO1Δ*paeII* (pJT19). [a] The different abundance of all transcripts in strain PAO1Δ*paeII*(pJTPaeII) and PAO1Δ*paeII*(pJT19) was verified by eye using the genome browser. A gene marked with (+) shows a clear difference, marked with (~) represenst a slight difference and marked with (-) represents no visible difference in transcript abundance. [b] The fold change represents the in- or decrease of the respective transcript levels in strain PAO1Δ*paeII*(pJTPaeII) when compared to strain PAO1Δ*paeII*(pJT19). Highlighted in gray are genes which form an operon. Genes which are potentially regulated by the transcriptional zinc uptake regulator Zur are also indicated.

In total, the transcript abundance of mRNAs belonging to nine operons were found to be affected upon *paeII* expression. For example the operon comprising genes PA5539 to PA5541 contains the two most up-regulated transcripts in the presence of PaeII, namely the genes PA5540 and *pyrQ* (PA5541). The afore mentioned *pyrQ* (also known as *pyrC2*) gene encodes a dihydrooratase which participates in pyrimidine nucleotide biosynthesis and it is required for growth in minimal medium (Brichta *et al.,* 2004). Furthermore, PA5540 encodes a protein which is predicted to be involved in phenylacetate catabolic processes. It shares sequence homologies with carbonic anhydrase which are zinc-containing enzymes. However, not only this operon but also other potential target genes of PaeII seem to be controlled by the transcriptional **z**inc **u**ptake **r**egulator, Zur (Table 2, marked with Zur). Although *zur* is the first gene in the three gene operon *zur-znuC-znuB*, only *znuB* is affected by PaeII expression. On the opposite strand a second operon reaching from PA5536 (*dksA2*) to PA5534 is located,

which is also regulated by Zur and affected by PaeII. The genes PA5334 and PA5335 are of unknown function. The transcription factor DksA2 is selectively expressed under Zn^{2+} limitation, it is a zinc-independent paralog of DksA (Furman *et al,.* 2013). As response towards stress conditions, DksA together with ppGpp inhibits transcription of rRNA genes and activates genes the function of which is required during poor nutrient conditions. Transcription of *dksA2* was shown to be controlled by the transcriptional repressor Zur (Ellison *et al.,* 2013). Furthermore, the bicistronic operon formed by the genes PA3600 and PA36001, which, according to the *Pseudomonas* database, are predicted to encode zinc-independent paraloges of ribosomal proteins, was shown to be up-regulated as a result of *paeII* transcription (Winsor *et al.,* 2011).

A third PaeII dependent operon (PA1922[*cirA*]-PA1925) entails *cirA* which is a probable TonB-dependent outer membrane receptor for ferrienterochelin and colicins (Hantke, 1990; Shirley and Lamont, 2009). Another TonB-dependent receptor encoded by PA0781 is also shown to be up-regulated by PaeII expression.

Apart from inducing several genes of unknown function, PaeII also up-regulates the stand alone genes *acsA, metE* and *osmE.* According to the *Pseudomonas* database, *metE* encodes a methionine synthase (Winsor *et al.,* 2011).

The acetyl-CoA synthetase AcsA is induced by substrates like ethanol and acetate. The response regulator ErdR controls *acsA* transcription (Kretzschmar *et al.,* 2010). However ErdR has not been identified as a potential target of PaeII in the present RNA-sequencing analysis.

It has been shown that *acsA* transcription is subjected to catabolite repression by succinate. Furthermore, it has been shown that *acsA* is negatively regulated by Hfq (Kretzschmar *et al.,* 2010; Sonnleitner *et al.,* unpublished).

An additional up-regulated gene is *osmE,* which encodes the osmotically responsive outer membrane lipoprotein OsmE (Firoved and Deretic, 2003). Another gene which was induced by PaeII is *amiA* (PA5538). AmiA acts as an amidase which plays a role in cell wall biogenesis.

Two software tools, TargetRNA (Tjaden, 2008) and RNAPredator (Eggenhofer *et al.,* 2011) were used to predict target mRNAs for the processed form of the sRNA PaeII. According to the bioinformatic analyses, none of the transcripts, which were affected by PaeII expression observed in the RNA-sequencing analysis, showed segments in their 5' UTR with

complementarity to PaeII (data provided in the appendix). This suggested, that the different abundances of transcripts observed in the presence and absence of PaeII, are not directly caused by binding of PaeII to these mRNAs.

The corresponding p-values of negatively expressed genes suggest that down-regulation is significant, but all observed fold-changes are rather low. Therefore, we considered this dataset as not significantly different. Out of the six down-regulated genes the function of three is unknown. *PhzB2* is part of a seven gene operon (*phzA1B1C1D1E1F1G1*) which, together with a second operon (*phzA2B2C2D2E2F2G2*), encodes genes required for pyocyanin and phenanzine biosynthesis (Mavrodi *et al.,* 2001). In addition, the genes coding for two outer membrane porins *oprD* and *oprE* are down-regulated (Choi *et al.,* 2011). Porins are needed for uptake of substances from the environment. OprD is a basic-amino-acid-specific porin, whereas OprE is an anaerobically induced porin predicted to be required for the uptake of either arginine or proline (Tamber *et al.,* 2006).

3. Discussion

3.1 Inhibition of *rpoS* translation by the sRNA PaeIII

The alternative σ-factor RpoS regulates gene expression through binding to specific promoter sequences. Together with the core RNA polymerase it initiates transcription. RpoS of *P. aeruginosa* is active in stationary phase during which it is involved in the general stress response. This σ-factor also controls the production of several extracellular virulence factors such as pyocyanin or exotoxin A (Fujita *et al.,* 1994; Suh *et al.,* 1999). In addition, *P. aeruginosa* RpoS promotes the formation of biofilms (Hogardt *et al.,* 2004). A microarray analysis performed by Schuster *et al.* (2004) defined the RpoS regulon in *P. aeruginosa.*
In stationary phase approximately 800 genes are controlled by RpoS, including many quorum-sensing controlled genes. In *E. coli* efficient *rpoS* translation is Hfq dependent (Muffler *et al.,* 1996). This is also true for *P. aeruginosa rpoS*, albeit to a lesser extent (Sonnleitner *et al.,* 2003).

As shown in Fig.23, over-expression of *paeIII* reduced the translation of *rpoS::lacZ* mRNA predominantly during early stationary phase. However, if the region spanning from nt +11 to +18 of PaeIII, which comprises the part complementary to the predicted binding site in *rpoS* mRNA that coincides with the Shine and Dalgarno sequence of *rpoS*, was deleted (Fig.20), the inhibitory effect on *rpoS::lacZ* translation was abrogated. To further investigate the putative interaction and to determine if base pairing is required for regulation, three mutations were introduced in the predicted interaction site of *paeIII* (PaeIIImut). The corresponding complementary nucleotides of *rpoS* (*rpoS*mut*::lacZ)* were also modified.
As shown in Fig.24B, the mutations in the PaeIIImut sRNA did not affect negative regulation of *rpoS::lacZ* translation by PaeIIImut. Furthermore, translation of the mutated *rpoS*mut*::lacZ* mRNA seemed to be less efficient when compared to the translation of *rpoS::lacZ* (Fig.24B/C). This is most probably due to the point mutations close to the ribosome binding site. Moreover, the wild type PaeIII sRNA was shown to repress *rpoS*mut*::lacZ* translation (Fig.24C).

As reported by Kawamoto *et al.* (2006) the *E. coli* sRNA Sgrs and the target *ptsG* mRNA share a 30 nt long complementary region. However, only a core portion of six nucleotides is responsible for SgrS action (Kawamoto *et al.,* 2006). Therefore, with regard to the interaction of PaeIII with *rpoS* mRNA, we concluded that altering only three bases of the PaeIII binding site is not sufficient to abolish the negative effect on *rpoS* translation.

As reported by Khakimova *et al.* (2013) RpoS is partly required for full expression of *katA*, which encodes catalase A. A *rpoS*$^{-}$ strain exhibited only 35% of the catalase activity observed in wild-type cells (Khakimova *et al.,* 2013). This is in agreement with a microarray study where *katA* was shown to be 2-fold down-regulated in a *rpos* mutant strain (Schuster *et al.,* 2004).

As observed in the microarray study conducted by Sorger-Domenigg (2010), *katA* was 2.62 fold down-regulated in the presence of PaeIII (Sorger-Domenigg, 2010). This could be attributable to the negative regulation of *rpoS* via PaeIII. *KatA* is the primary constitutive catalase expressed in the absence of H_2O_2 induction, whereas *katB* expression is known to be induced only during exogenous H_2O_2 stress (Khamikowa *et al.,* 2013). Hence, in a stress free environment the main catalase activity can be attributed to KatA. Therefore it seems worth testing whether PaeIII has an indirect influence on *katA* expression.

3.2 The sRNA PaeII affects transmembrane transport

Bacterial sRNAs enable the cell to respond to various environmental changes by modulating gene expression (Gottesman and Storz, 2011). Regulation of gene expression by sRNAs can be achieved by direct base-pairing to target mRNAs which may cause mRNA destabilization and degradation. The corresponding changes in the levels of individual transcripts can be monitored by highthroughput RNA sequencing analyses.
In the present study we performed a RNA-sequencing analysis and compared the transcriptome profile of a *P. aeruginosa* strain expressing the sRNA PaeII and a *paeII* mutant strain. At a glance, the results indicated that PaeII seems to affect transmembrane transport functions (Fig.27; Table 2).

In *E. coli* translation of the *cirA* mRNA is blocked by binding of Hfq near the ribosome binding site. Consequently, Hfq causes degradation of the *cirA* mRNA. Pairing of the sRNA RyhB to *cirA* mRNA activates translation by promoting structural changes, thereby relieving Hfq binding to *cirA* mRNA which in turn prevents RNA degradation (Salvail *et al.,* 2013). It can be speculated that a similar mechanism might also exist in *P. aeruginosa* since upon over-expression of *paeII, cirA* mRNA levels are 17 fold increased. Hfq is known to bind preferentially to AU-rich regions adjacent to stem-loop structures. Such a region is present in the PAO1 *cirA* mRNA (nt -20 to -15; `AUAAU)` **.**

The data further suggest that PaeII might be involved in zinc dependent gene regulation. Among the possible PaeII targets some are known to be regulated by the *P. aeruginosa* zinc uptake regulator Zur, which controls zinc uptake in a zinc responsive manner. For example, the zinc specific ABC-transport system ZnuABC and also the zinc independent transcriptional regulator DksA2 was known to be repressed by Zur (Blaby-Haas *et al.,* 2011; Ellison *et al.,* 2013). Upon expression of *paeII* an up-regulation of *dksA2* and *znuB* was observed.
Graham *et al.* (2009) reported that upon zinc depletion in *E. coli, ykgM* was up-regulated. This gene encodes the non Zn^{2+} ribbon-containing paralogue of the ribosomal protein L31 which is predicted to bind Zn^{2+}, and it was shown that *ykgM* expression is regulated by *E. coli Z*ur (Panina *et al.,* 2003). The genes PA3600 (ribosomal protein L36 type b) and

Pa3601 (ribosomal protein L31 type b) form an operon. They are deemed to encode zinc independent paralogs of *P. aeruginosa* ribosomal proteins and are regulated by Zur.
These two genes were shown to be up-regulated as a result of *paeII* transcription.
As *zur* translation is not influenced by PaeII expression, the sRNA action on Zur dependent genes might be indirect.

Another possible indirect regulation caused by PaeII concerns the 2.17 fold up-regulated gene *acsA,* which was also shown to be negatively regulated by Hfq (Sonnleitner, unpublished). However, studies by Sonnleitner showed that PaeII binds to Hfq with low affinity. Therefore, it is rather unlikely that sequestration of Hfq by PaeII, which might cause *acsA* up-regulation, is the underliying regulatory mechanism.

4. Material and Methods

4.1 Bacterial strains, plasmids and growth conditions

The bacterial strains used in these studies are listed in Table 3. Bacterial cultures were grown at 37°C in different growth media. To investigate the role of PaeIII, the cells were grown in Luria-Bertani (LB) medium (Miller, 1972), whereas studies concerning PaeII were performed in BM2 minimal medium supplemented with succinate (20mM).

Antibiotics were added to a final concentration of 100µg/ml tetracyclin and and 400µg/ml kanamycin for *P. aeruginosa.* For *E. coli* 100µg/ml ampicillin, 50µg/ml kanamycin and 30µg/ml tetracyclin were used. For induction of RNA overexpression from plasmids toluate (2mM) was used.

Plasmids and oligonucleotides used in this study are listed in Table 4 and 5, respectively.

Table 3

Strain	Genotype/Relevant Features	Reference
P. aeruginosa		
PAO1	wild-type	(1)
PAO1Δ*paeII*	in frame *paeII* deletion	(2)
PAO1Δ*paeIII*	in frame *paeIII* deletion	(2)
PAO1*rpoS*$^-$	PAO1*rpoS101::aacCI, GmR*	(4)
PAO1*anr*$^-$	in frame *anr* deletion	(5)
E. coli		
*DH5*α	*recA1 endA1 hsdR17 thi-1 supE44 gyrA96 relA1 deoRΔ(lacZYA-argF) U169 (Φ80lacZΔM15)*	(3)
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*	

(1) Holloway *et al.*, 1979; (2) Sorger-Domenigg, 2010; (3) Sambrook *et al.,* 2001; (4) Suh *et al.,* 1999; (5) Vallet-Gely *et al.,* 2007;

Table 4

Plasmid	Genotype / Relevant Features	Reference
pJT19	plasmid carrying inducible *Pm/xylS* promoter, Kan^R	(1)
pJTPaeIII	pJT19 harbouring *paeIII*	(2)
pME6014	cloning vector for translational *lacZ* fusions, Tc^R	(3)
pME6014*rpoS*	translational in frame fusion of the first 56 codons of *rpoS* to *lacZ*	(2)
$pJTPaeIII_{\Delta 11\text{-}18}$	pJT19 harbouring *paeIII* with a deletion spanning nt 11-18	this study
pJTPaeIIImut	pJT19 harbouring *paeIII* with three mutations: nt +4 G→A, nt +7 G→A, nt +8 T→C with regard to TSS	this study
pME6014*rpos*mut	translational in frame fusion of the first 56 codons of *rpoS* to *lacZ.* It contains three point mutations in *rpoS* nt -1 C→T, nt -4 C→T, nt -5 A→G with regard to the translational start site	this study
pUC19	ColE1 replicon; Ap^R	(4)
pJTPaeII	pJT19 harbouring *paeII*	this study

(1) Winther-Larsen *et al.,* 2000; (2) Sorger-Domenigg, 2010; (3) Heeb *et al.,* 2000; (4) Norrander *et al.,* 1983, nucleotide numbers are with respect to the A (+1) transcriptional (PaeIII) and A (+1) translational (*rpoS*) start site.

Table 5

Name	Sequence 5‘ – 3‘ [a]	Reference	Binding Region [b]
I26	CCCCACACTACCATCGGCGATGCGTCG	(1)	6039619-6039645
V28	CCCCACACTACCATCGGCGATGCGTCG	(1)	4523929-4523905
W28	CGTGCGGCACGGTGGTGCGGG	(1)	3958114-3958094
V40	TTTTTTTT**ACTAGT**CCAGCGCTGTACTATCCCTTCC (*SpeI*)	(1)	3958051-3958072
J40	TTTTAAAA**GGTACC**GAAAAGGTGAGCCCCGGAGGT (*KpnI*)	(1)	3958150-3958131
A52	AAAA**GGATCC**GTTCCACCCGCAGTTAGTACG (*BamHI*)	(1)	4059497-4059477
B52	TTTT**CTGCAG**GCGTGTAGTCGATGTGCTTGTG (*PstI*)	(1)	4058742-4058763
O52	AAAA**ACTAGT**TCCTTCACGAGCCCTCGGCT (*SpeI*)	this study	4523841-4523861
P52	AAAA**GGATCC**GGTTGTGCGGAGGCATTAA (*BamHI*)	this study	4523948-4523966
P71	TTTTTTTT**ACTAGT**AGCGCTGTAC/CCAGCGCTGATCGCGACCC (*SpeI*)	this study	3958053-3958063 3958071-3958090
V85	TTTTTTTT**ACTAGT**CAGCACTACACTATCCCTTCC *(SpeI*)	this study	3958052-3958072
W85	GCAAGGGATAGTGATATGGCACTCAAAAAAG	this study	4057894-4057925
X85	CTTTTTTGAGTGCCATATCACTATCCCTTGC	this study	4057894-4057925
Z96	AAAAAAAAAAAAAAAAAAGCGTTTCACTTCTGAGTTCGGCA	(2)	binding to 5s rRNA

[a] bold letters represent restriction sites and the respective name of the restriction enzyme is bracketed, [b] numbers show binding sequence on PAO1 chromosome according to the P*seudomonas* genome database, http://www.pseudomonas.com ; (1) Sorger-Domenigg *et al.,* 2010; (2) Gómez-Lozano *et al.,* 2012;

4.3 Construction of plasmids

A plasmid for overexpression of *paeII* was constructed as follows. The *paeII* gene was amplified by PCR using the primers O52/P52 and chromosomal DNA of PAO1 as template. The 108-bp PCR fragment was cleaved with *SpeI* and *BamHI* and then ligated into the corresponding sites of plasmid pJT19. The resulting plasmid pJTPaeII harbors *paeII* under transcriptional control of a *Pm/xylS* promoter (Table 4).

A plasmid for overexpression of *paeIII* with a deletion spanning from nt +11 to +18 with respect to the A (+1) of the transcriptional start was constructed as follows. This sequence of PaeIII was predicted to be involved in interaction with *rpoS* RNA (Fig.18, *). The $paeIII_{\Delta 11\text{-}18}$ variant was amplified by PCR using the primers P71/J40 and chromosomal DNA of PAO1 as template. The 90-bp PCR fragment was cleaved with *SpeI* and *KpnI* and then ligated into the corresponding sites of plasmid pJT19. The resulting plasmid pJTPaeIII$_{\Delta 11\text{-}18}$ harbors $paeIII_{\Delta 11\text{-}18}$ under transcriptional control of a *Pm/xylS* promoter (Table 4).

A plasmid for overexpression of *paeIII* haboring three point mutations within the putative interaction site for *rpoS* was constructed as follows (Fig.24A). Site-directed mutagenesis was carried out with PCR using oligonucleotides that contained the desired mutations. The mutant *paeIII* gene was amplified by PCR using the primers V85 (mutation primer)/J40 and chromosomal DNA of PAO1 as template. Nucleotides +4, +7 and +8 with regard to the A (+1) of the transcriptional start site were changed from A**UG**UC**G**CGA to A**CA**UC**A**CGA. The 98-bp PCR fragment was cleaved with *BcuI* and *SpeI,* and then ligated into the corresponding sites of plasmid pJT19. The resulting plasmid pJTPaeIIImut harbors the *paeIII*mut variant*,* which is under transcriptional control of a *Pm/xylS* promoter (Table 4).

A translational fusion between the *rpoS::lacZ* gene with three point mutations in the putative interaction site of *rpoS* for PaeIII (Fig.24A) was constructed as follows. A 755nt long DNA fragment consisting of the *rpoS* promoter and the first 56 codons was amplified by PCR using the primers A52/B52 and chromosomal DNA of PAO1 as template. The obtained fragment was cleaved with *BamHI* and *PstI* and cloned into the corresponding sites of plasmid pUC19. The resulting plasmid pUC*rpoS* was transformed into *E. coli XL1-blue* (Table 1). The mutant *rpoS* fragment was amplified by PCR using the primer W85/X85 and pUC*rpoS* DNA as template. Nucleotides -1, -4 and -5 with regard to the A (+1) of the start codon of *rpoS* were changed from A**AC**GA**C**AUG to A**GT**GA**T**AUG. The PCR produced intact pUC19 plasmid. Next, a restriction digest using *DpnI*, which cleaves methylated DNA, was performed. Thereby the methylated template DNA was removed. The pUC*rpoS*mut plasmid was cleaved with *BamHI* and *PstI* to isolate the *rpoS*mut DNA fragment, which was subsequently cloned into the corresponding sites of the translational *lacZ*-fusion plasmid pME6014. The resulting translational *lacZ*-fusion plasmid pME6014*rpoS*mut harbors a portion of *rpoS* with point mutations in nucleotides -1, -4 and -5 with regard to the A (+1) of the start codon of *rpoS* (Table 4).

All DNA manipulations were verified by DNA sequencing.

4.4 β-Galactosidase assay

The β-galactosidase assay was done as described by Miller (1972). *P. aeruginosa* cells were grown in 20ml LB medium (in 100ml Erlenmeyer flasks) with shaking at 37°C. For determination of the β-galactosidase activity, the cells were harvested by centrifugation (2min at 13krpm), resuspended in 1ml 0,9% NaCl and then lysed by addition of 40µl chloroform and 7,5µl 1% SDS. 10µl of the cell lysate were mixed with 990µl Z-Buffer and incubated for 3min at 37°C. Then, the reaction was started with addition of 200µl ONPG solution (4mg/ml). To stop the reaction 500µl 1M Na_2CO_3 was added. The results were expressed in Miller Units which were calculated using the following equation: $1000*OD_{420}*(dilution) / t(min) *V(ml)*OD_{600}$

4.5 RNA isolation

Total RNA was isolated using the hot phenol method as described by Leoni *et al.* (1996). The cells were harvested by centrifugation. The cell pellet was resuspended in 50 µl DEPC treated ddH_2O and mixed with preheated (65°C) 250µl lysis buffer and 500µl phenol (pH 5,5) and vigorously vortexed. After centrifugation (5' at 13,2k rpm) the aequous phase was extracted with an equal volume of phenol/chloroform (1:1), re-centrifuged and then extracted with an equal volume of chloroform. Then, the RNA was precipitated over-night at -20°C, by addition of 0,1 volume of 3M Na-acetate (pH 5,5) and 2,5 volumes of 96% EtOH. Removal of DNA was achieved by multiple rounds of DNase (Roche) treatment followed by phenol chloroform extraction and Na-acetate/ethanol precipitation.

4.6 Northern-blot analyses

Total RNA was isolated using the hot phenol extraction method and contaminating DNA was removed. Equal volumes of RNA loading dye and total RNA solution were mixed and incubated at 65°C for 5min to denaturate secondary RNA structures. RNA samples were loaded onto a 8% polyacrylamide/8M urea gel and electrophoretically separated. The RNA was transferred to a nylon membrane (Hybond N, GE-Healthcare) by electroblotting and the RNA was UV crosslinked to the nylon membrane. The membrane was kept in hybridization buffer for one hour at 55°C. Then, gene specific ^{32}P-end labeled-oligonucleotides were added (PaeII: V28, PaeIII: W28, 5s control: I26; Table 3) and hybridization was performed over-night. The hybridization signals were visualized with a PhosphorImager (Molecular Dynamics).

4.7 Transcriptomics next generation RNA-sequencing

High throughput RNA-sequencing provides a measure of the presence and prevalence of transcripts from each gene (Mortazavi *et al.,* 2008). The strains PAO1Δ*paeII*(pJT19) and PAO1Δ*paeII*(pJTPaeII) were grown in triplicates to an OD_{600} of 1.0 in 20ml BM2 medium supplemented with succinate (20mM). Then, *paeII* overexpression from plasmid pJTPaeII (Table 4) was induced by addition of 2mM toluate. After 30min, 10ml samples were taken. Total RNA was extracted using the hot phenol method and contaminating DNA was removed by DNase (Roche) treatment. To deplete the samples from ribosomal RNAs a modified version of the MICROBEExpress Kit (Ambion) was used. In addition to the already provided oligonucleotides specific for 23s and 16s rRNA, also oligonucleotides specific for 5s rRNA (Z96; Table 5) were added. For the following cDNA synthesis the RNA was fragmented to a length of 100-400 nucleotides. The randomly primed double stranded cDNA was then synthesized using the SuperScript Double-Stranded cDNA Kit (Invitrogen). The concentration of double stranded cDNA was determined using a Nanodrop Fluorospectrometer. The cDNA library was prepared using the NEBNext DNA Sample Prep Master Mix Set1. Ready to use adaptors were provided by *the CSF (Campus Science Support Facilities GmbH, csf.ac.at)*. Depletion of unligated adaptors was achieved by size selective gel purification. The samples were then UDGase digested (enables strand specificity) which was followed by a PCR to enrich the adaptor ligated cDNA. Next generation single end sequencing was performed at the *CSF NGS Unit* using an illumina Hiseq 2000 sequencer. The obtained sequencing reads were mapped to the *Pseudomonas aeruginosa* PAO1 genome using the software *segemehl* (Hoffmann *et al.,* 2009). The sequencing data was transferred to the GenomeBrowser which depicts the number of reads for a given genome segment. Due to technical reasons of the six initial samples only four, two of each strain, were used for further analyses. A bioinformatic differential gene expression analysis was performed using the software *DEseq* (Anders and Huber, 2010). Genes with adjusted p-values smaller or equal to 0.05 were determined to be differentially expressed (Table 2).

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6. Appendix

6.1 Buffers and solutions

Media

1l LB:

10g Peptone
5g Yeast extract
10g NaCl
Add dH_2O to final volume of 1l
pH = 7

1l BM2

40mM K_2HPO_4
22mM KH_2HPO_4
7mM $(NH)_2SO_4$
0,5mM $MgSO_4$
0,01mM Fe SO_4
20mM Sugar (succinate)
Add dH_2O to final volume of 1l

β-Galactosidase assay

1l Z-Buffer:

16,1g $Na_2HPO_4x7H_2O$
5,5g $NaH_2PO_4xH_2O$
0,75g KCl
0,246g $MgSO_4x7H_2O$
Add ddH_2O to final volume of 1l
Before usage add β-Mercaptoethanol 2,7ml/l

Substrate

4mg/ml ONPG solution

Stop Solution

1M Na_2CO_3

RNA Extraction

Lysis Buffer:
2% SDS
30mM Na-Acetate
3mM EDTA pH 8
Adjust pH to 5,5

Northern-blot analyses

Use 8% PAA 8M Urea Gel

1l 10xTBE Buffer
108g Tris
55g boric acid
7,5g EDTA
Add ddH_2O to final volume of 1l

1l 20x SSC Buffer
175,3g NaCl
88,7g sodium citrate
Adjust to pH7 with NaOH
Add ddH_2O to final volume of 1l

Hybridization solution II
4ml 2% BSA solution
4ml 2% PVP solution
4ml 2% Ficoll solution
4ml 0,5M EDTA
50ml 20x SSC buffer
2ml 20% SDS solution
Add DEPC treated ddH_2O final volume of 200ml
Before use add 2g Dextran 500 and 200µl salmon sperm DNA (10mg/ml)

1l Washing buffer 1
100ml 20x SSC
5ml 20% SDS
Add ddH_2O to final volume of 1l

1l Washing buffer 2
5ml 20x SSC
5ml 20% SDS
Add ddH_2O to final volume of 1l

6.2 Bioinformatic target prediction for the processed form of PaeII

Rank	Gene	z-Score	Binding region on the mRNA	Binding region on the sRNA
2	PA4681	-4.66	0 / +17	+7 / +24
11	PA1398	-2.88	+11 / +37	+7 / +34
14	PA1238	-2.79	-46 / -35	+5 / +16
20	PA3279	-2.69	+25 / +33	+38 / +46
38	PA4311	-2.45	+30 / +38	+8 / +16
77	PA0680	-2.19	+9 / +19	+6 / +16
78	PA0040	-2.19	+32 / +40	+8 / +16
83	PA3220	-2.16	-24 / -9	+2 / +16
97	PA3896	-2.11	-24 / -11	+2 / +16

The webtool RNApredator was used to identify potential targets of the processed form of sRNA PaeII (http://rna.tbi.univie.ac.at/RNApredator2/target_search.cgi). Only those interactions were considered which are predicted to be located within the region spanning from nt -50 to +50 on the mRNA. The location of the predicted interaction site on the sRNA and mRNA is noted. Nucleotides are with respect to the A(+1) of the translational start site of the mRNA or with respect to the A(+1) of the transcriptional start site of the sRNA, respectively.

Rank	Gene	Score	Pvalue	Binding region on the mRNA	Binding region on the sRNA
1	PA4681	-78	0.00041831	+17 / -1	+7 /+24
2	PA1238	-70	0.0017332	-36 / -49	+5 / +18
3	PA0544	-67	0.00295254	-14 /-50	+8 / +46
4	PA3659	-67	0.00352594	-7 / -25	+12 / +30
5	sodB	-64	0.00600273	+2 / +33	+1 / +36
6	PA4220	-63	0.00600273	-5 / +14	+8 / +28
7	trpI	-62	0.00716636	+1 / +17	+8 / +23
8	PA5001	-62	0.00716636	-15 / -32	+14 /+31
9	ubiA	-62	0.0085546	+13 /+35	+7 / +26
10	PA0861	-61	0.0085546	+18 / +37	+9 / +27
11	PA1398	-61	0.0085546	+12 / +38	+7 / +34
12	PA1923	-61	0.0085546	-13 / +20	+6 / +36
13	PA5033	-61	0.0085546	+12 / +29	+7 / +24

The webtool TargetRna was used to identify potential targets of the processed form of sRNA PaeII (http://snowwhite.wellesley.edu/targetRNA/).
Only those interactions were considered which are predicted to be located within the region spanning from nt -50 to +50 on the mRNA.The location of the predicted interaction site on the sRNA and mRNA is noted. Nucleotides are with respect to the A(+1) of the translational start site of the mRNA or with respect to the A(+1) of the transcriptional start site of the sRNA, respectively.

6.3 Results of a microarray analysis performed by Sorger-Domenigg

Gene ID[a]	P-Value	Fold Change[b]	Target Description	Functional class
PA0740	0,0017	5,57	SdsA1, probable beta-lactamase	Putative enzymes
PA4770	0,0923	2,13	lldP, L-lactate permease	Transport of small molecules
PA3516	0,004	-8,07	probable lyase	Putative enzymes
PA3519	0,001	-7,43	hypothetical protein	Hypothetical, unclassified, unknown
PA3518	0,017	-7,00	hypothetical protein	Hypothetical, unclassified, unknown
PA3515	0,016	-5,02	hypothetical protein	Hypothetical, unclassified, unknown
PA3517	0,038	-4,35	probable lyase	Carbon compound catabolism
PA3520	0,000	-2,24	hypothetical protein	Hypothetical, unclassified, unknown
PA3523	0,122	-2,05	probable RND[c] efflux membrane fusion protein precursor	Transport of small molecules

Sorger-Domenigg (2010) compared the transcriptom profiles of strains PAO1 and PAO1Δ*paeII* by use of a microarray assay. The strains were grown in minimal medium supplemented with mannitol (20mM). The potential targets of PaeII as observed in the microarray study are shown above.

Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen and well known for its ability to colonize almost any environment, which requires a quick adaption of the bacterial physiology. The majority of the studied *Pseudomonas aeruginosa* small regulatory RNAs (sRNAs) appear to adjust bacterial physiology in response to environmental changes or stress conditions and are involved in the regulation of virulence genes. There are three major classes of small regulatory RNAs. They can exert their regulatory function by base-pairing with mRNAs, by interaction with proteins or by targeting of DNA as exemplified by the CRISPR defense system. The group of sRNAs which act by base-pairing with mRNAs frequently requires the RNA chaperone Hfq for their function.

Previous studies identified two novel *Pseudomonas aeruginosa* sRNAs, which are termed PaeII and PaeIII, respectively. The sRNA PaeII is encoded between open reading frames PA4040 and PA4041 and is 108nt in length. It is processed to a shorter ~70 nucleotide RNA by RNase E. Expression of *paeII* was shown to be massively induced throughout growth of *P. aeruginosa* in minimal medium. To further elucidate the function of PaeII a high-throughput RNA sequencing analysis was performed with strain *P. aeruginosa* PAO1 and with an isogenic strain harboring a deletion in the *paeII* gene.

The results showed that expression of *paeII* resultend in a different abundance of trancripts coding for proteins involved in transmembrane transport. The possible targets of PaeII included several genes regulated by the *P. aeruginosa* zinc uptake regulator Zur.

The sRNA PaeIII is expressed during stationary phase. A transcriptome analysis indicated that *rpoS* mRNA translation is impeded by PaeIII. A bioinformatic analysis revealed a possible interaction site of PaeIII with *rpoS* mRNA, which includes the Shine and Dalgarno sequence. The comparison of the β-Galactosidase levels conferred by a translational *rpoS::lacZ* fusion confirmed that PaeIII impedes translation of the *rpoS* mRNA and the negative regulation involves a part of the predicted PaeIII-*rpoS* interaction site.

Zusammenfassung

Pseudomonas aeruginosa ist ein humanpathogenes Bakterium und bekannt dafür eine Vielzahl verschiedener Lebensräume zu besiedeln. Essentiell dafür ist die schnelle Adaptierung der bakteriellen Physiologie in Reaktion auf sich ändernde Umweltbedingungen. Die meisten bis jetzt erforschten kleinen regulatorischen RNAs in *P. aeruginosa* sind Teil der Mechanismen die zu einer Anpassung der Genexpression an die äußeren Bedingungen führen. Kleine regulatorische RNAs können an mRNAs binden und dadurch die Translation beeinflussen.

Vorhergegangene Studien identifizierten zwei neue kleine *P. aeruginosa* (PAO1) RNAs, die PaeII und PaeIII benannt wurden. Die 107 Nukleotide lange sRNA PaeII ist zwischen den offenen Leserahmen PA4040 und PA4041 kodiert. Diese RNA wird kontinuierlich unter Nährstoff limitierenden Bedingungen synthetisiert. PaeII wird durch RNase E zu einer kürzeren, 70 Nukleotide langen, Variante prozessiert. Um die mögliche Funktion von PaeII zu ergründen wurde eine Transkriptomanalyse durchgeführt. Hierzu wurde der Gesamtpool an mRNA in *P. aeruginosa* PAO1 und einer isogenen *paeII* Mutante sequenziert.

Es konnte gezeigt werden, dass die Expression von PaeII vor allem mRNAs beeinflusst die für Transmembran Transportsysteme kodieren. Eine weitere Gemeinsamkeit einiger PaeII regulierten Gene ist, dass sie unter der Kontrolle des Regulators der Zink-Aufname, Zur, stehen.

PaeIII wird in der stationären Wachstumsphase von PAO1 produziert. Eine vorangegangen Transkriptomanalyse indizierte, dass die Expression von *rpoS* durch PaeIII reduziert wird. Bioinformatische Analysen sagten eine mögliche Interaktionsstelle von PaeIII mit der *rpoS* mRNA, im Bereich der Shine and Dalgarno Sequenz, voraus. Die *rpoS* Translation war im *P. aeruginosa* Wild Typ Stamm gegenüber der PAO1 *paeIII* Mutante reduziert.

Die negative Regulation von *rpoS* mRNA mittels PaeIII konnte durch Deletion der PaeIII Sequenz, die mit der *rpoS* mRNA interagiert, untermauert werden.

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Curriculum vitae

Stephan Rindler

Schulbildung

Mai 2005	Abschluss mit Matura
1997 – 2005	Bundesrealgymnasium Spittal/Drau, Kärnten Naturwissenschaftlicher Schwerpunkt
1993 – 1997	Volksschule Spittal/Drau bzw. Lieserhofen, Kärnten

Studium

April 2014	Abschluss Masterstudium Molekulare Mikrobiologie
März 2012	Masterarbeit am Department Mikrobiologie, Immunbiologie & Genetik, unter Prof.Dr. Udo Bläsi
März 2011	Abschluss Bachelorstudium Biologie mit Schwerpunkt Mikrobiologie & Genetik, unter Prof.Dr. Angela Witte
Oktober 2006 – März 2011	Bachelorstudium Biologie mit Schwerpunkt Mikrobiologie & Genetik

Zusätzliche Qualifikationen

Im Zuge des Master und Bachelorstudiums erlernte ich grundlegende Methoden der Chemie, der Immun- sowie Zellbiologie und vertiefende Methoden der molekularen Mikrobiologie. Während meines Master und Bacherlorstudiums half ich verschiedene praktische und theoretische Kurse, als Tutor, zu betreuen.