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1 Introduction

1.1 Gammaproteobacteria

The phylum Proteobacteria is the largest within the domain of Bacteria and contains a wide range of organisms with metabolically distinct lifestyles. Because of its enormous versatility, the phylum was named after the greek sea god Proteus, who was able to perform polymorphous shape-shifting (Stackebrandt, 1988). The Proteobacteria can be divided into the six main classes α, β, γ, δ, ε and ζ (Woese, 1987; Stackebrandt, 1988). These classes contain phototrophic, chemoorganotrophic and chemolithotrophic bacteria. The cell morphology ranges from rod-like, coccoid and spiral to pleomorph shapes. The class Gammaproteobacteria contains about 250 genera and is the biggest class that consists solely of Gram-negative bacteria (Garrity *et al.*, 2005). Therefore, this class is by itself bigger than most other phyla in the kingdom of Bacteria. The Gammaproteobacteria include many plant, animal and human pathogens like *Legionella, Salmonella, Yersinia* and *Pseudomonas* (Figure 1).

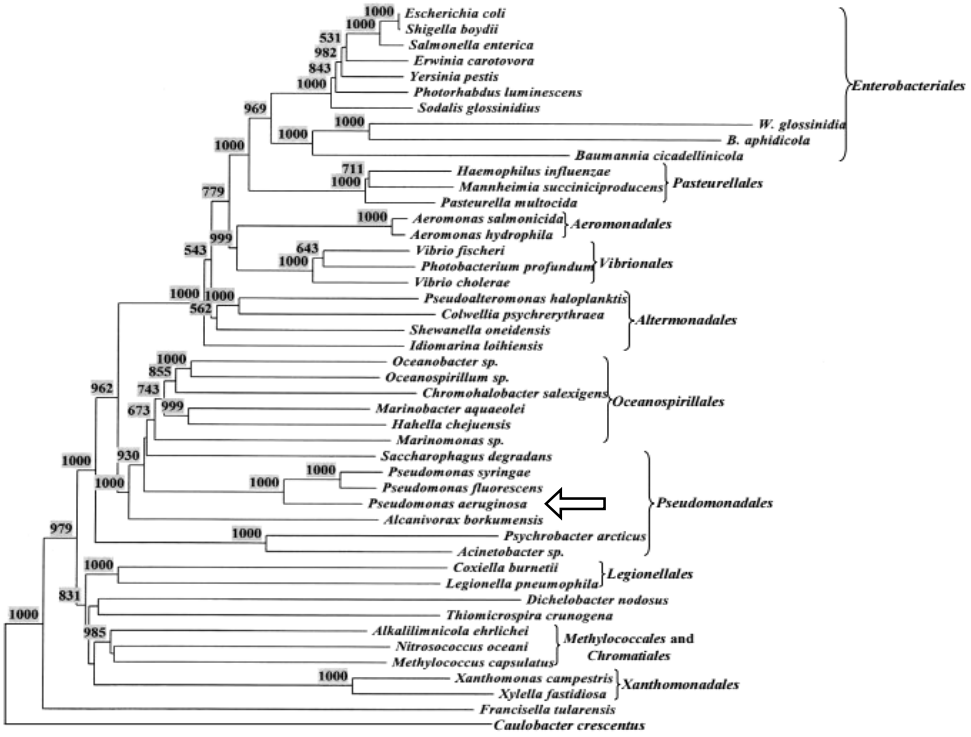


Figure 1. A neighbour-joining distance tree of the class Gammaproteobacteria.

Gao *et al.* (2009) analyzed the evolutionary relationships of Gammaproteobacteria based on the concatenated sequences for 36 highly conserved proteins. The Gammaproteobacteria comprise the largest amount of genera with an enormous versatility concerning their phenotypic properties. *P. aeruginosa* is marked with an arrow. Figure adapted from Gao *et al.* (2009).

1.2 *Pseudomonas aeruginosa*

The versatile Gammaproteobacterium *Pseudomonas aeruginosa* is found in soil and water, however, it can also colonize plants, animals and humans (Gupta *et al.*, 2013). The cells are motile and react to gradients of attractants and repellants by chemotaxis (Masduki *et al.*, 1995). *P. aeruginosa* is an opportunistic pathogen that hardly affects healthy people, but has medical relevance as a nosocomial pathogen (Cross *et al.*, 1983). It is the third most frequent bacterium behind Staphylococci and Enterococci in causing antimicrobial-resistant infections in hospitalized patients. Particularly persons suffering from severe burns or cystic fibrosis are afflicted with serious infections (Gibson *et al.*, 2003). In addition, hospitalized people are prone to transmittance *via* catheterization, lumbar punctures, tracheostomy and intravenous infusions (Madigan *et al.*, 2010). *P. aeruginosa* is highly adaptable and apart from a respiratory metabolism, it is also capable of growing anaerobically with nitrate or nitrite as electron acceptors. *P. aeruginosa* can use a wide range of organic compounds as a carbon source. Compared to other Gammaproteobacteria, *P. aeruginosa* has a rather large genome (6.25 Mb) (Stover et al. 2000). This genetic set up enabled the development of complex regulatory circuits that permit the adaption to several environments.

1.2.1 Bacterial communities: Quorum-sensing and biofilm

Bacteria can adapt their physiology in relation to the population density by quorum-sensing (QS). This mechanism was initially described in *Vibrio fischeri,* where bioluminescence is controlled by the lux system (Nealson *et al.*, 1970). *P. aeruginosa* quorum-sensing employs four different cell-to-cell signaling molecules: two acyl-derivatives of homoserine lactone, a 2-heptyl-3-hydroxy-4-quinolone, termed the Pseudomonas quinolone signal (PQS) and its precursor 2-heptyl-4-quinolone (HHQ). These quorum-sensing systems regulate the expression of many virulence factors in response to the density of the bacterial culture (Pesci *et al.*, 1997). The Las system (Figure 2) is composed of the autoinducer synthase LasI, the autoinducer molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and the transcriptional regulatory protein LasR (Pearson *et al.*, 1994). The Rhl system (Figure 2) employs a similar mechanism and consists of the RhlR transcriptional regulatory protein, the autoinducer *N*-butyryl-L-homoserine lactone (C_4-HSL) and the synthase RhlI (Pearson *et al.*, 1995). The autoinducer homoserine lactone (AHL) is produced by the synthase and binds to the regulatory protein, resulting in its activation. Only at high cell densities the AHL levels are sufficient to activate transcription of QS-regulated genes *via* the transcriptional regulatory proteins. Another part of the quorum-sensing system involves the secondary metabolites PQS and HHQ (Figure 2).

PQS is an intercellular signaling molecule that controls the expression of many virulence genes (Pesci *et al.*, 1999). The last step of PQS synthesis is the conversion of HHQ to PQS, mediated by the putative monooxygenase PqsH (Deziel *et al.*, 2004). The precursor HHQ can function as an autoinducer molecule even without conversion to PQS (Figure 2) (Diggle *et al.*, 2007). Both molecules contribute to the pathogenicity of *P. aeruginosa via* attenuation of the innate immune system of host cells (Kim *et al.*, 2010). The transcriptional regulator PqsR (MvfR) positively influences the production of PQS (Cao *et al.*, 2001). Thus, the *pqs* operon is stimulated by PqsR and encodes genes for the biosynthesis of HAQs (4-hydroxy-2-alkylquinolines) like HHQ and PQS (Figure 2). PqsR is an important regulator of the QS circuitry, as it is required for the synthesis of several virulence genes that control anthranilic acid biosynthesis (Deziel *et al.*, 2005) and other precursors of the virulence factor pyocyanin. Pyocyanin is a pigment that creates reactive oxygen species, which cause oxidative stress in the tissue of the host (Lau *et al.*, 2004). It has been shown, that QS regulates genes required for infection and host interactions, such as exoproteases, siderophores, exotoxins and many secondary metabolites. These molecules are also required during chronic infection (Davies *et al.*, 1998).

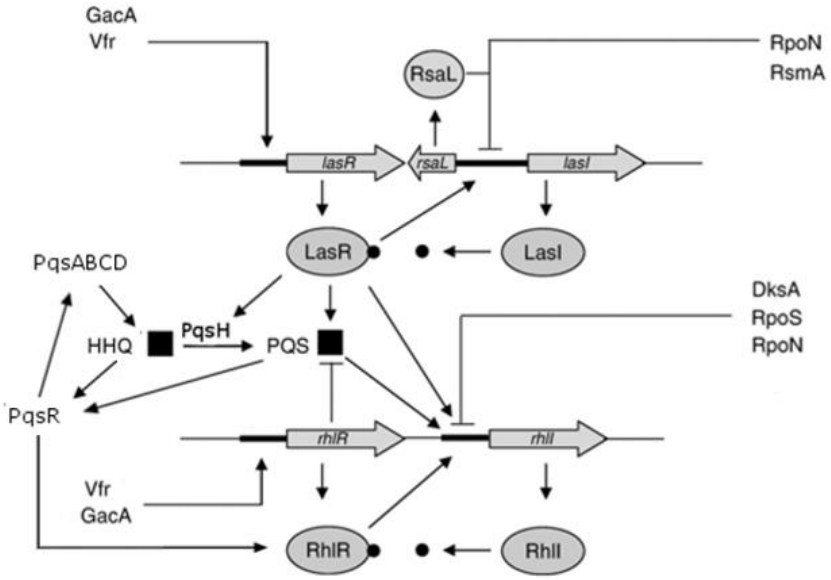


Figure 2. Quorum-sensing in *P. aeruginosa*.

Various signals activate the transcription of genes encoding the regulatory proteins LasR and RhlR. LasI and RhlI synthesize the autoinducer molecules 3-oxo-C_{12}-HSL and C_4-HSL, respectively, which are necessary for cell-to-cell communication. The transcriptional regulators act together with the cognate autoinducers to activate Rhl and Las-controlled genes. Moreover, the Pseudomonas quinolone signal PQS serves as a regulatory link between the Las and the Rhl system. In addition, it acts as an autoinducer molecule by itself, just like the PQS precursor 2-heptyl-4-quinolone (HHQ). The putative monooxygenase PqsH converts HHQ to PQS (Deziel *et al.*, 2004). Upon binding of HHQ or PQS to the transcriptional regulator PqsR, PqsR stimulates transcription of many genes necessary for PQS production and pyocyanin synthesis. Image modified from Venturi (2006).

Furthermore, the QS system impacts on antibiotic susceptibility and biofilm formation (Wagner *et al.*, 2003). In their natural environment bacteria are often associated in biofilm communities. Biofilm formation is a complex process, where planktonic cells attach to a surface in response to environmental signals. The cells proliferate and form microcolonies that develop to a mature biofilm structure (Costerton *et al.*, 1999). This biofilm matrix is able to colonize the lungs of cystic fibrosis (CF) patients (reviewed by Moreau-Marquis *et al.* (2008)), but it can also attach to inanimate surfaces like urinary catheters, polystyrene or contact lenses (Nickel *et al.*, 1985; Miller and Ahearn, 1987). In CF patients, non-mucoid *P. aeruginosa* infections are frequently detected in small infants, which turn into persistent mucoid forms when antibiotic treatment does not start in early childhood. Almost 80% of all young adults suffering from cystic fibrosis have already developed persistent *P. aeruginosa* biofilms in their lungs. This is a major cause of morbidity in CF patients, as in many cases the treatment of persistent *P. aeruginosa* infections is futile (Heijerman, 2005). For instance, sessile *P. aeruginosa* cells are one order of magnitude more resistant to tobramycin than free-swimming cells (Nickel *et al.*, 1985). Such infections cannot be treated without an even distribution of antibiotic concentrations throughout the whole biofilm. However, the cells are protected by exopolysaccharides and other extracellular components, which hinder diffusion of antimicrobials (Gordon *et al.*, 1988). Upon insufficient treatment, the persistent *P. aeruginosa* cells become even more recalcitrant (Hoyle and Costerton, 1991). Thus, an antibiotic therapy kills the free-swimming population without any detrimental effect on the biofilm itself. This frequently leads to recurring infections (Marrie *et al.*, 1982). Moreover, biofilms are a heterogenous matrix, where the cells are in different growth stages. Within the biofilm they have to cope with various conditions like oxygen, iron or nutrient limitation. Starving cells are growing more slowly, and therefore are less susceptible to antibiotics, which mostly target cell division and metabolism (Brown *et al.*, 1988). Hence, biofilm structures exert many strategies to evade the immune system and antimicrobial therapy, therefore their treatment remains challenging.

1.2.2 Shift to chronic infection: The Gac/Rsm pathway

In CF patients and immunocompromised persons, the transition from an acute non-mucoid *P. aeruginosa* infection to a persistent mucoidy state is observed very frequently. This switch between acute and chronic infection is governed by the GacS/GacA (global antibiotic and cyanide control) two-component system (Goodman *et al.*, 2004). This system was first described in the soil bacterium *Pseudomonas fluorescens* (Laville *et al.*, 1992). Two-component systems sense extracytoplasmic signals *via* a sensor histidine kinase, which autophosphorylates a histidine upon signal binding and transfers the phos-

phoryl group to the corresponding receiver domain. This response regulator (RR) stimulates transcription of target genes in its phosphoylated state (Hoch, 2000). Stover *et al.* (2000) identified 55 two-component sensors and 89 RRs within the *P. aeruginosa* strain PAO1, most of which are still uncharacterized. This vast amount of additional regulatory mechanisms may be one reason for the versatility of *P. aeruginosa.* The Gac/Rsm signal transduction pathway promotes a chronic state of infection, resulting in a sessile lifestyle, activation of quorum-sensing and production of extracellular components. The response regulator GacA activates transcription of the two protein-binding sRNAs RsmY and RsmZ, which target the post-transcriptional regulator RsmA (regulator of secondary metabolism) (Figure 3 and Figure 10) (Kay *et al.*, 2006; Brencic *et al.*, 2009). RsmA belongs to the CsrA/ RsmE family and impedes translational initiation *via* binding to GGA-rich regions in the 5' untranslated region (UTR) of target mRNAs (Schubert *et al.*, 2007; Lapouge *et al.*,

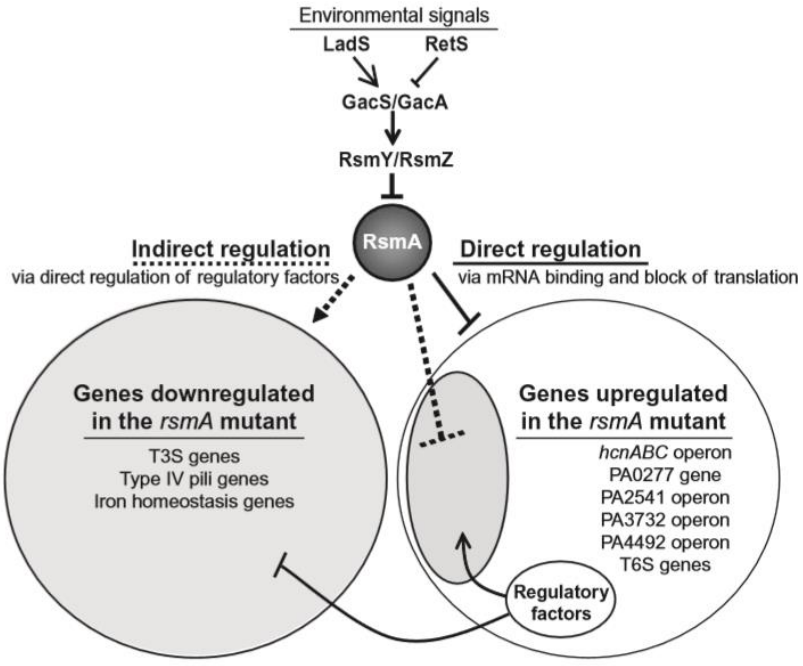


Figure 3. Effects of the post-transcriptional regulator RsmA.

When RsmA is not titrated by the regulatory sRNAs RsmY and RsmZ, it binds to GGA motifs upstream of the start codon of target mRNAs, thereby blocking ribosome-binding (Schubert *et al.*, 2007; Lapouge *et al.*, 2007). A microarray analysis showed that this affects operons associated with secondary metabolism (extracellular factor HCN, Exoenzyme S) or type VI secretion (Brencic and Lory, 2009). In addition, several transcription factors are direct RsmA targets in *P. aeruginosa* (Burrowes *et al.*, 2006; Brencic and Lory, 2009), which leads to indirect activation of type III secretion, type IV pili and iron homeostasis genes. Many genes are also indirectly down-regulated by RsmA *via* a regulatory factor. Arrows, positive regulation; orthogonal lines, negative regulation; solid lines (white background), direct control; dotted lines (grey background), indirect control. Scheme adapted from Brencic and Lory (2009).

2007). It negatively regulates secondary metabolism, quorum-sensing and biofilm formation (Figure 2 and Figure 10) (Pessi and Haas, 2001; Pessi *et al.*, 2001; Reimmann *et al.*, 1997). Besides, RsmA positively influences type III secretion, swarming motility and iron homeostasis (Figure 3). However, it is likely that this is an indirect effect *via* repression of transcriptional regulators (Heurlier *et al.*, 2004; Brencic and Lory, 2009). RetS and LadS are two hybrid sensor kinases that impact on the Gac/Rsm regulatory network (Figure 3). RetS directly interacts with GacS and inhibits its function (Goodman *et al.*, 2009). LadS on the other hand, appears to stimulate GacS function (Ventre *et al.*, 2006; Chambonnier *et al.*, 2016).

1.2.3 Adaption to starvation: The stringent response

Adaption to harsh environmental conditions is crucial for survival of the bacterial population. Therefore, Bacteria have developed elaborate mechanisms to cope with various stresses like starvation, antimicrobial agents or lack of oxygen. During growth in a nutrient-depleted environment, an adaptive mechanism known as the stringent response is activated by the signaling molecule 3′,5′-bispyrophosphate (ppGpp). In rich medium, the levels of ppGpp and pppGpp (guanosine 3′-diphosphate,5′-triphosphate) are constant, whereas starvation leads to their accumulation in the cytoplasm (Cashel and Gallant, 1969). The ppGpp and pppGpp levels are controlled by the two enzymes RelA and SpoT (Chatterji and Ojha, 2001). The alarmone ppGpp binds the β and β´-subunits of the RNA polymerase (RNAP) (Artsimovitch *et al.*, 2004) and inhibits transcription from ribosomal promoters (Paul *et al.*, 2004a). In addition, ppGpp can promote direct activation of amino acid biosynthesis promoters (Paul *et al.*, 2005) and indirect activation of alternative sigma factors through increased competitiveness of the core RNAP (Jishage *et al.*, 2002). In *E. coli,* this is accomplished in concert with the protein DksA, an accessory factor of ppGpp, which binds to the RNAP holoenzyme and shortens open complex half-life during transcription inititation (Paul *et al.*, 2004b; Perederina *et al.*, 2004). Together with ppGpp, DksA can also facilitate and accelerate open complex formation, leading to enhanced transcription (Paul *et al.*, 2005). This dependence of transcriptional regulation on ppGpp and DksA is also operative in *P. aeruginosa* (Perron *et al.*, 2005). Moreover, DksA is involved in regulatory networks like quorum-sensing (Figure 2) (Branny *et al.*, 2001) and virulence factor production (Jude *et al.*, 2003). The canonical DksA protein contains a zinc-finger motif, which is important for structure maintenance and activity, whereby four cysteine residues are binding to a Zn^{2+}-ion (Webb *et al.*, 1999; Perederina *et al.*, 2004). Zinc is essential for basic cellular processes like DNA replication and is part of many metalloenzymes, however, above certain concentrations this element is toxic for the cell (Bruins *et al.*, 2000; McDevitt *et al.*, 2011). Therefore, the intracellular concentration has

to be tightly controlled (Silver, 1996). Mammalians reduce the zinc availability within infected tissue as a defensive measure, which is why zinc-independent paralogs of proteins may confer an evolutionary advantage during host infection (Kehl-Fie and Skaar, 2010; Botella *et al.*, 2011).

DksA2 is a zinc-independent, functional paralog of DksA, which is present in *P. aeruginosa* (Haas *et al.*, 2009). The two proteins share only 34% sequence identity (Furman *et al.*, 2013) and DksA2 is missing the zinc-finger motif. Nevertheless, DksA2 can compensate for the DksA function during zinc deficiency (Blaby-Haas *et al.*, 2011). DksA2 has RNAP-binding affinity and influences transcription levels of ribosomal and amino acid biosynthesis genes, respectively. In *P. aeruginosa,* a putative zinc uptake regulator (Zur) binding site (Pederick *et al.*, 2015) was identified in the 5' UTR of the *dksA2* gene (Figure 20), suggesting a role in zinc homeostasis (Blaby-Haas *et al.*, 2011; Furman *et al.*, 2013). The negative transcriptional regulation of Zur relies on successful binding of zinc-ions to the M and D sites of this Fur-like regulator. Thus, under zinc depletion Zur is not able to bind its targets and repression is relieved (Figure 4) (Smith *et al.*, 2009). Zur controls transcription of the *znuABC* operon, which encodes the high affinity zinc importer ZnuABC (Figure 4) (Ellison *et al.*, 2013). ZnuABC consists of the zinc-binding periplasmic protein ZnuA, an inner membrane permease (ZnuB) and the ATPase ZnuC (Patzer and Hantke, 1998).

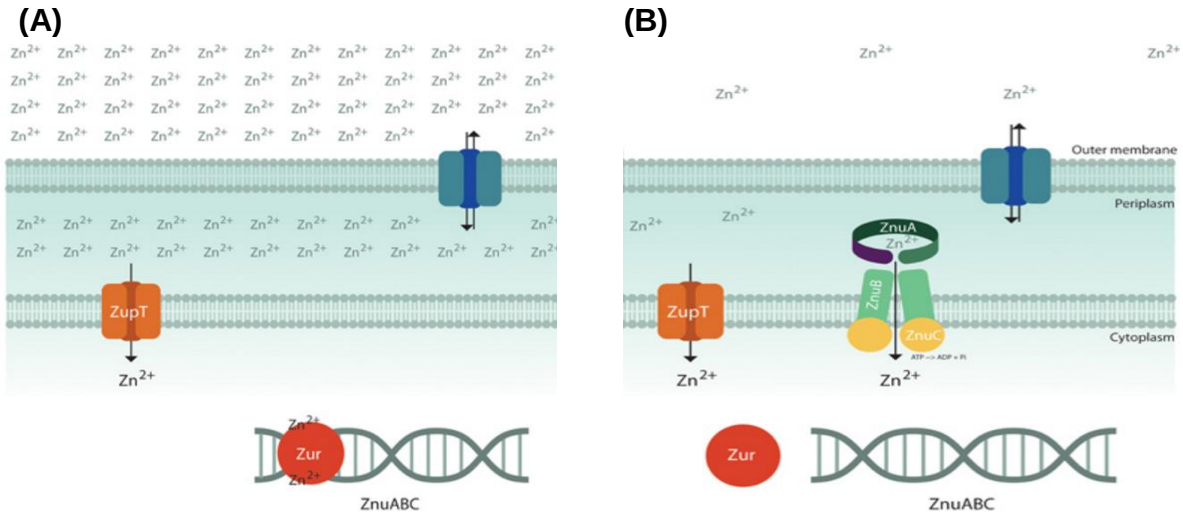


Figure 4. Zinc uptake in Gram-negative bacteria.

Zinc uptake from the environment is regulated by Zur. The bacterial outer membrane is permeable for zinc ions. (**A)** As long as the zinc levels are high, the low affinity zinc transporter ZupT, which is located in the cytoplasmic membrane, is sufficient and the zinc uptake regulator Zur is bound to DNA, leading to repression of the *znuABC* gene cluster. (**B)** Upon zinc depletion, no zinc ions are bound to Zur, repression is relieved and the genes encoding the high affinity zinc importer ZnuABC are expressed. Hence, ZnuABC is able to sustain the zinc concentration together with ZupT. Figure adapted from Cerasi *et al.* (2013).

1.2.4 Expression on demand: Alternative sigma factors

Bacterial transcription is conducted by the RNAP, which consists of the core enzyme with five subunits (α_2, β, β´, ω). The association with different σ subunits forms the RNAP holoenzyme. The holoenzyme can bind the promoter DNA and commence transcription initiation, which requires a series of conformational changes to mediate open complex formation (Gruber and Gross, 2003). RpoD, the σ^{70} housekeeping sigma factor, is primarily responsible for transcription during the exponential phase of growth (Fujita *et al.*, 1994; Lonetto *et al.*, 1992). In addition to RpoD, 23 other sigma factors that belong to the sigma 70 class are known i.e. RpoH (σ^{32}), RpoF (σ^{28}) and RpoS (σ^{38}), as well as the extracytoplasmic-acting sigma factors AlgU (RpoE, σ^{22}), PvdS and SigX, respectively (Potvin *et al.*, 2008). The only sigma factor that is inherently different from canonical sigma 70 factors is RpoN (formerly NtrA, σ^{54}), which was originally identified in *E. coli* (Hirschman *et al.*, 1985; Hunt and Magasanik, 1985). The RpoN promoter region contains a highly conserved -12 GC motif and a -24 GG motif, together with a ~ 10 nucleotide spacer in between (T**GG**CAC-N5-TT**GC**) (Shingler, 1996). RpoN has a sophisticated way of controlling expression from its target promoters that shows resemblance to eukaryotic polymerase II (Wang *et al.*, 1992; Shingler, 1996). Binding of the σ^{54} holoenzyme at a cognate promoter region leads to formation of a stable closed complex, that is not prone to leaky expression (Buck et al., 2000). Activation of transcription initiation only takes place when a certain bacterial enhancer-binding protein (EBP) binds to an upstream activator sequence (UAS) located 80 - 150 bp upstream of the promoter region. The formation of a DNA loop enables binding of the EBP to the RNAP and this initiates open complex formation (Bush and Dixon, 2012). This mechanism guarantees tight control without a repressor protein. Upon activation by environmental or metabolic signals, high expression levels can be achieved instantly (Buck *et al.*, 2000; Collado-Vides *et al.*, 1991; Wang *et al.*, 1998). A comprehensive mRNA profiling analysis in *P. aeruginosa* by Schulz *et al.* (2015) revealed that 680 genes are within the RpoN regulon, thus being the largest regulon of all alternative sigma factors. Indeed, many cellular processes have been reported to be influenced by RpoN; first and foremost nitrogen assimilation (Hirschman *et al.*, 1985; Gussin *et al.*, 1986), but also quorum-sensing, mucoid colony morphology, attachment, motility and chemotaxis (Ishimoto and Lory, 1989; Totten *et al.*, 1990; Mattick *et al.*, 1996; Boucher *et al.*, 2000; Thompson *et al.*, 2003; Heurlier *et al.*, 2003). Because of this wide range of functions, RpoN requires a large number of different EBPs (Table 1). This σ^{54}-dependent family of regulator proteins contains a central domain for interaction with the RNAP, a C-terminal domain with a DNA-binding motif and an optional N-terminal domain common in two-component response regulators (RR) (Stock *et al.*, 1989; Morett and Segovia, 1993). This N-terminal domain is the regulatory domain of the RR proteins, which interacts with phosphorylated histidine protein kinases (West and Stock, 2001).

Several EBPs have been described in *P. aeruginosa*: PilR regulates type 4 pili production (Ishimoto and Lory, 1992), AlgB is involved in exopolysaccharide production (Wozniak and Ohman, 1991), PhhR takes part in L-phenylalanine and L-tyrosine catabolism (Song and Jensen, 1996), and FleQ controls flagellar gene regulation (Table 1) (Jyot *et al.*, 2002). CbrB, the RR of the CbrA/B two-component system, and NtrC, the RR of the NtrB/C two-component system, react to variations in nitrogen and carbon levels and adapt the catabolism according to the needs of the cell (Nishijyo *et al.*, 2001). In addition, NtrC plays a role during nitrogen limitation (Stock *et al.*, 1989; Kustu *et al.*, 1991; Fischer, 1994).

Table 1. Examples of bacterial enhancer-binding proteins.

EBP	Function	Response regulator	Organism	Reference
AlgB	Alginate production	yes	*P. aeruginosa*	Wozniak and Ohman (1991)
PilR	Type 4 pili	yes	*P. aeruginosa*	Ishimoto and Lory (1992)
CbrB	Carbon-nitrogen balance	yes	*P. aeruginosa*	Nishijyo *et al.* (2001)
NtrC	Nitrogen assimilation and fixation	yes	*P. aeruginosa*	Li and Lu (2007)
DmpR	Catabolism of phenol and methylphenols	no	*P. putida*	Shingler *et al.* (1993)
XylR	Catabolism of aromatic hydrocarbons	no	*P. putida*	Inouye *et al.* (1987)
PhhR	L-phenylalanine and L-tyrosine	no	*P. aeruginosa*	Song and Jensen (1996)
	catabolism		*P. putida*	Ng *et al.* (1995)
FleQ	Flagellar gene regulation	no	*P. aeruginosa*	Jyot *et al.* (2002)

The sigma factor RpoS (σ^{38}) was first described in *E. coli* as a stationary phase sigma factor important during nutrient starvation (Lange and Hengge-Aronis, 1991). In *P. aeruginosa, rpoS* expression increases steadily with growth and reaches the highest levels upon entry into stationary phase (Fujita *et al.*, 1994). It has been shown in *P. aeruginosa,* that σ^{38} plays a role in the production of extracellular virulence factors (alginate, pyocyanin, exotoxin A), is involved in quorum-sensing (Figure 2) (Latifi *et al.*, 1996) and increases defense mechanisms during cell stress induced by low pH, heat, high osmolarity, hydrogen peroxide or ethanol (Venturi, 2006). Moreover, Whiteley *et al.* (2001) reported that RpoS negatively influences biofilm formation and resistance to the antibiotic tobramycin. Albeit σ^{38} has similar functions in *E. coli* and *P. aeruginosa*, in *E. coli* many RpoS-regulated genes are involved in cell division, adaption and protection, whereas in *P. aeruginosa,* this is only the case for a small set of genes (Venturi, 2003; Schuster et al., 2004). The regulation of *rpoS* is complex and poorly understood in Pseudomonads (Figure 4). In *P. fluorescens,* the two-component system GacS/GacA positively regulates

rpoS transcription. *GacA* and *gacS* mutants are expressing less than a fifth of the wild-type levels at the onset of the stationary phase (Whistler *et al.*, 1998). RpoS has no influence on RsmA in *P. fluorescens*, but *rpoS* was shown to be repressed by RsmA (Heeb *et al.*, 2005). In addition, the DNA-binding protein PsrA activates *rpoS* transcription *via* binding to a conserved sequence comprising the palindromic motif C/GAAC-N2-4-GTTG/C (Kojic *et al.*, 2002). PsrA belongs to the TetR familiy of regulatory proteins, which control their own expression by a negative feedback loop. In *P. aeruginosa* and *P. putida, psrA* mutants display only 50% of the wild-type RpoS levels in stationary phase (Kojic and Venturi, 2001). PsrA can enhance or repress transcription of its target genes depending on the binding region near the promoter sequence (Venturi, 2003). In *P. aeruginosa*, overproduction of the stringent response effector RelA leads to increased *rpoS* transcription in exponential phase and decelerates cell growth in general (van Delden *et al.*, 2001).

In enteric bacteria, three Hfq-dependent small RNAs are positive regulators of *rpoS* translation, DsrA, RprA and ArcZ. All three sRNAs bind within a hairpin stem-loop that reduces translational efficiency, but they are differentially regulated. *RprA* transcription is activated by the RcsC/B phosphorelay system (Majdalani *et al.*, 2002), whereas *dsrA* transcription is enhanced at low temperatures (Sledjeski *et al.*, 1996). ArcZ is activated under aerobic

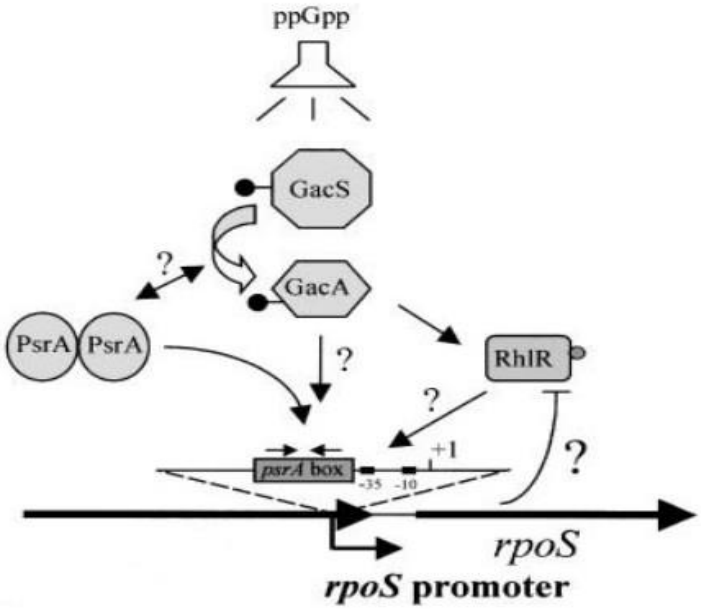


Figure 4. Putative regulatory mechanism of *rpoS* transcription in Pseudomonads.
The transcriptional regulator PsrA binds to the *rpoS* promoter region and enhances *rpoS* transcription. Up to now, it remains unknown if the stimulatory effect of the Gac/Rsm signal transduction pathway is direct or indirect. GacA has a positive effect on the quorum-sensing regulatory protein RhlR, which might also increase *rpoS* transcription levels. On the other hand, it is possible that the quorum-sensing system is negatively influenced by RpoS *via* RhlR. The stringent response inducer molecule ppGpp plays a role in activating *rpoS* transcription dependent on RelA. Figure adapted from Venturi (2003).

growth conditions and is repressed by the ArcA/B two-component system during anoxic growth (Papenfort *et al.*, 2009; Mandin and Gottesman, 2010). The sRNA OxyS also requires Hfq for function, but has a negative effect on *rpoS* expression. OxyS is induced by oxidative stress and represses *rpoS* translation (Zhang *et al.*, 1998). Until now, no corresponding regulatory sRNAs have been identified in *P. aeruginosa.*

1.2.5 The RNA-binding protein Hfq

The Hfq protein was originally described as a host factor for RNA bacteriophage Qβ replication (Franze de Fernandez *et al.*, 1968). This protein is required for the regulatory function of most *trans*-encoded sRNAs in Proteobacteria. Hfq belongs to the Sm/LSm protein family, members of which are also present in Eukaryotes and Archaea (Schumacher et al. 2002). In *E. coli*, Hfq controls its own expression *via* autoregulation (Vecerek *et al.*, 2005). The abundance of *E. coli* Hfq increases during growth and reaches levels almost as high as those observed for ribosomal proteins. In a fast growing *E. coli* culture this corresponds to approximately 10,000 Hfq hexamers/cell (Valentin-Hansen *et al.*, 2004). In *P. aeruginosa* on the other hand, the intracellular Hfq concentration was determined with ~ 2,200 hexamers per cell (Sonnleitner and Bläsi, 2014). The *P. aeruginosa* Hfq protein can compensate an *E. coli hfq* mutation in terms of *rpoS* regulation and Qβ replication, which corroborates its highly conserved function (Sonnleitner *et al.*, 2002). The deletion of *the hfq* gene in *P. aeruginosa* is associated with reduced virulence, less oxidative stress tolerance and an impaired twitching and swarming motility (Sonnleitner *et al.*, 2003). X-ray crystallography of the *E. coli* and *P. aeruginosa* Hfq proteins revealed a donut-shaped structure that consists of homohexamers (Sauter *et al.*, 2003; Nikulin *et al.*, 2005). In *E. coli*, the proximal side of the protein complex was found to interact with U-rich sequences, whereas the distal side has a high affinity for single-stranded A-rich motifs (Mikulecky *et al.*, 2004).

Owing to its RNA chaperone activity, Hfq can modulate RNA secondary structure, leading to conformational changes (Moll *et al.*, 2003a). Furthermore, Hfq fulfills another criterion essential for a genuine RNA chaperone; it has the ability to facilitate the interaction between two complementary RNA sequences (Afonyushkin *et al.*, 2005). This has been shown in *E. coli* for binding of the Spot42 sRNA to the *galK* mRNA (Moller *et al.*, 2002). Binding of Hfq to a sRNA may lead to target repression by inhibiting translation (Figure 5A) (Udekwu *et al.*, 2005) or resolving a secondary structure at the ribosome-binding site (RBS), and thereby enabling translational initiation (Figure 5C) (Majdalani *et al.*, 1998). Furthermore, Hfq is able to shield sRNAs from RNase E-dependent cleavage (Figure 5E) (Masse *et al.*, 2003; Moll *et al.*, 2003b) or favor degradation of sRNA-mRNA duplexes (Figure 5B) (Masse *et al.*, 2003; Pfeiffer *et al.*, 2009). The latter mechanism has been stu-

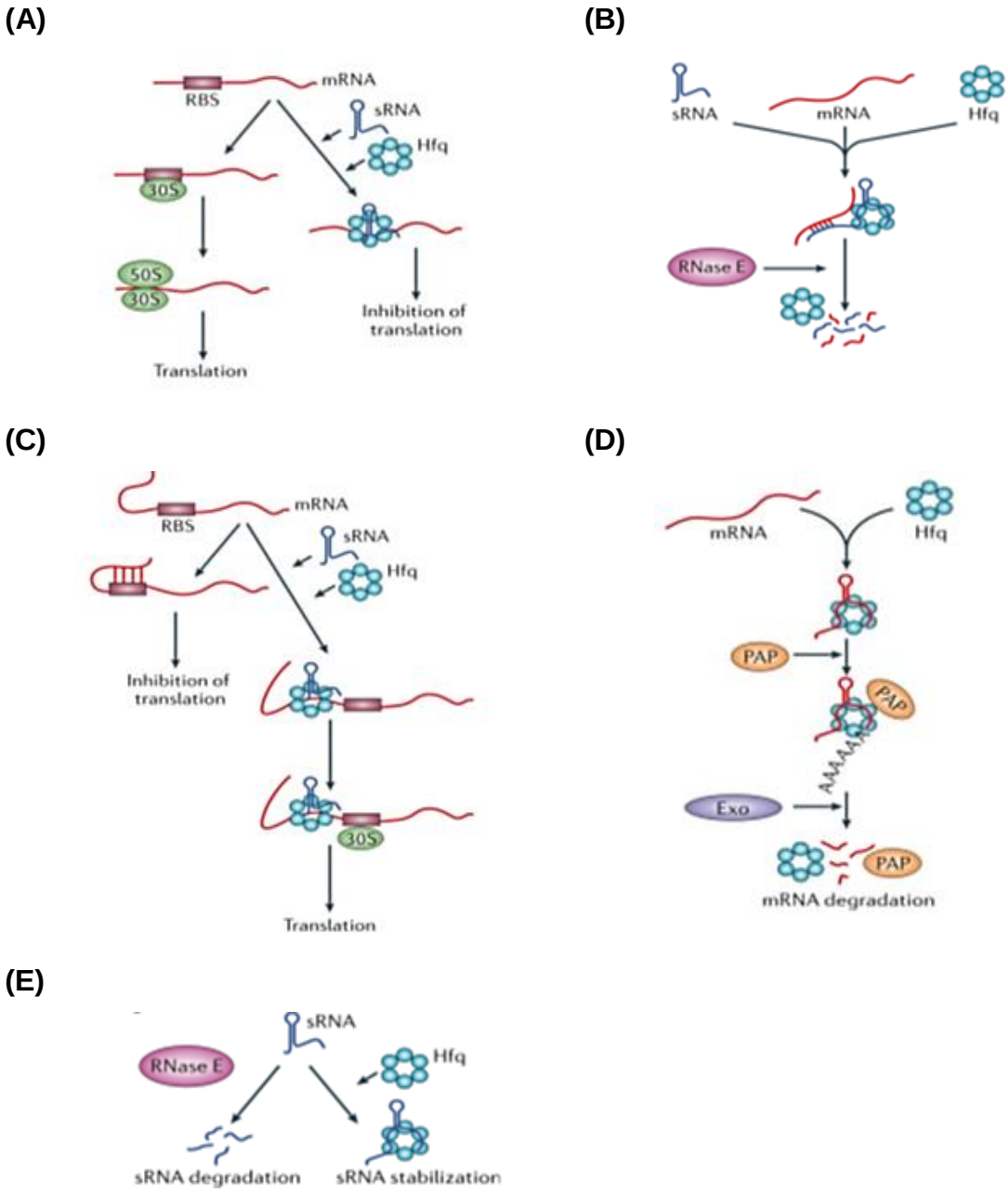


Figure 5. Different functions of Hfq concomitant with a sRNA.

(A) Translational repression: Hfq sequesters, together with a sRNA, the RBS of a target gene and 30S binding is impeded. **(B)** RNA destabilization: Binding of Hfq and a sRNA can reveal an RNase E cleavage site on the mRNA, leading to the degradation of the sRNA-mRNA duplex. **(C)** Translational activation: The RBS of a target mRNA is masked by a secondary structure in the 5' UTR. A sRNA-Hfq complex can bind in this region and resolve the secondary structure, leading to translational initiation. **(D)** RNA degradation: Hfq-binding to an mRNA can result in polyadenylation by poly(A) polymerase (PAP), which is followed by exoribonuclease (Exo)-mediated degradation. **(E)** RNA stabilization: Hfq-binding to a sRNA can protect from RNase E cleavage, thereby increasing the stability of the sRNA. Further explanation is provided in the text. Figure adapted from Vogel and Luisi (2011).

died for the *E. coli* sRNA RyhB, which binds, for instance, to the *sodB* mRNA. This leads to Hfq-mediated degradation of the RyhB-*sodB* complex in an RNase E dependent manner (Masse *et al.*, 2003). In addition, Hfq has the ability to recruit poly(A) polymerase (PAP), which results in 3' - 5' degradation of the poly (A) tail by an exoribonuclease (Figure 5D) (Mohanty *et al.*, 2004; Hankins *et al.*, 2010). However, not all *trans*-acting sRNAs require Hfq for their activity. VrrA, a sRNA discovered in the gammaproteobacterium *Vibrio cholerae*, does not dependend on Hfq for negative regulation of the membrane protein gene *ompA* (Song *et al.*, 2008).

Moreover, Hfq can act as a direct translational repressor of target mRNAs. In *P. aeruginosa*, Hfq represses the translation of several genes that are involved in carbon catabolism (see Figure 9). For instance, Hfq was shown to bind *via* its distal side to the Shine-Dalgarno sequence of the *amiE* mRNA and thereby directly prevents translation initiation (Sonnleitner and Bläsi, 2014).

1.3 Regulatory RNAs in Bacteria

Regulatory RNAs mediate the response to diverse physiological conditions of the cell by interfering with transcription, translation or stability of mRNAs by distinct mechanisms (Waters and Storz, 2009). Thus, they represent a universal concept of bacterial gene regulation with hundred or more putative members in each organism (Wagner and Romby, 2015). The first chromosomally encoded small RNA, which has been discovered was the *E. coli* MicF transcript. The 174 bp *trans*-acting sRNA prevents translation of the *E. coli ompF* gene by base-pairing with the translation initiation region (Mizuno *et al.*, 1984). Depending on their mechanisms of action, regulatory RNAs are divided into five major subgroups: riboswitches, thermosensors, CRISPR RNAs, protein-binding and base pairing sRNAs.

Riboswitches are metabolite sensors that mediate conformational changes upon ligand-binding (small molecules). This leads to termination, anti-termination, translation or translational inhibition. They are part of the 5' UTR of mRNAs and consist of an aptamer region and an expression platform (Nudler and Mironov, 2004; Mandal and Breaker, 2004; Montange and Batey, 2008).

Thermosensors are *cis*-acting RNA thermometers (RNAT) that sense an increase in temperature. Secondary structures within the ribosome-binding site are transiently relieved at high temperatures and translation of virulence (37°C) or heat shock (≥ 40°C) mRNAs can proceed (Righetti and Narberhaus, 2014).

The **CRISPR RNAs** (clustered regularly interspaced short palindromic repeats) are a common mechanism in Bacteria and Archaea to combat phage infection. They confer

acquired immunity against foreign DNA or RNA by RNA-guided cleavage of nucleic acids from viruses, plasmids, prophages or transposons (Horvath and Barrangou, 2010).

Protein-binding sRNAs sequester proteins with regulatory functions and modulate their activity. The *E. coli* 6S RNA binds to RNAP holoenzymes with canonical σ^{70} factors and behaves like DNA during open complex formation (Figure 6A). This occurs in stationary phase, when the expression of genes regulated by alternative sigma factors is favored (Wassarman, 2007). Alternatively, protein-binding sRNAs can use structural mimicry of mRNAs, DNA or other sRNAs to titrate the protein (Figure 6B). CsrA and its homologues RsmA and RsmE are RNA-binding proteins, that are bound and inhibited by CsrB/RsmY family sRNAs (Lapouge *et al.*, 2008).

Base pairing sRNAs can either be *cis*- or *trans*-encoded. ***Cis*-encoded base pairing RNAs** are transcribed from the opposite strand and are fully complementary to their target mRNA. Although they originate from the same DNA region, *cis*-encoded sRNAs are distinct molecules and act in *trans* (Waters and Storz, 2009). Most *cis*-encoded sRNAs are

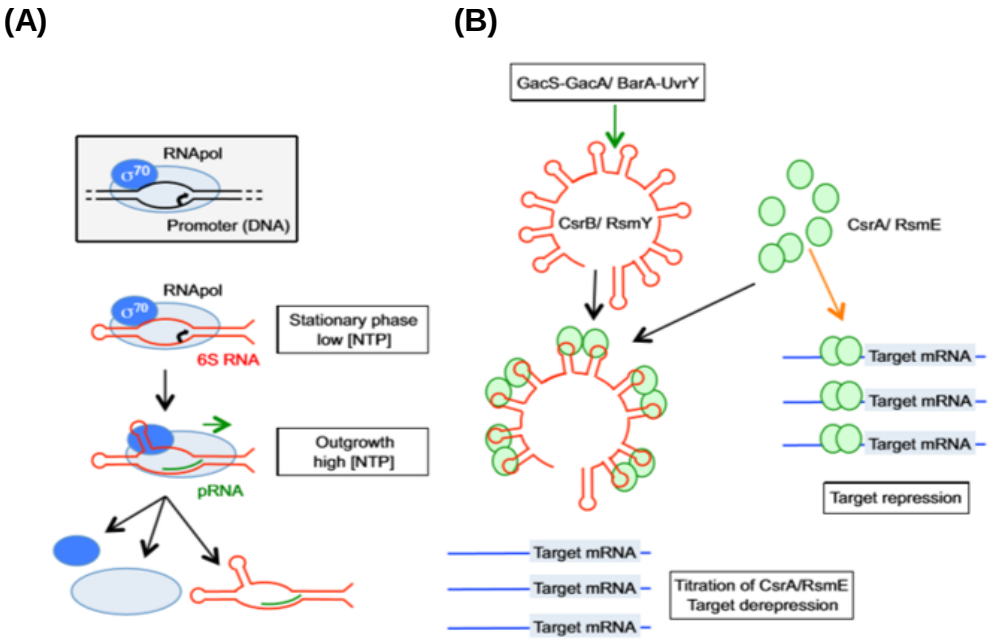


Figure 6. Modes of action employed by protein-binding sRNAs.

Protein-binding sRNAs influence the activity of RNA-binding proteins (RBPs) by imitating the structure of their RNA targets. This can include protein inhibition, as demonstrated for CsrB-like family members, or modification and regulation, as is the case for 6S RNA in *E. coli.* **(A)** At low nuleotide triphosphate (NTP) concentrations, the 6S RNA mimics an open promoter complex and this leads to binding of the RNAP. When the cells grow out of low NTP states, the pRNA binds to the 6S RNA and induces a structural change, resulting in release of the RNAP (Wassarman, 2007). **(B)** The CsrB/RsmY family sRNAs are induced by a two-component system and act *via* sequence-specific binding to CsrA/RsmE proteins. This titrates CsrA/RsmE away from their target mRNAs, followed by target de-repression (Lapouge *et al.*, 2008). Figure adapted from Wagner and Romby (2015).

transcribed from transposons, bacteriophages or plasmids and keep control of mobile genetic elements (Wagner *et al.*, 2002; Brantl, 2007). Chromosomal homologues of these elements, and their antisense sRNAs, have been described in many bacteria, and are often antitoxins that down-regulate the expression of their cognate toxin gene (Kawano *et al.*, 2007; Fozo *et al.*, 2008). In most cases, binding blocks translation and directs mRNA processing, which in turn results in mRNA cleavage (Wagner *et al.*, 2002; Brantl, 2007). Alternatively, some antisense sRNAs have the ability to stabilize transcripts which would otherwise be degraded (Gottesman and Storz 2011). A novel variation of transcriptional attenuation has been reported in *Shigella flexneri*. The *cis*-acting antisense sRNA RnaG binds to the nascent transcript of *icsA,* encoding a factor that promotes host colonization. This alters the secondary structure of the mRNA, which leads to the formation of an intrinsic terminator sequence and premature transcriptional termination (Figure 7F) (Giangrossi *et al.*, 2010).

***Trans*-encoded base pairing sRNAs** are transcribed from a genomic location that is not linked to the location of their target genes. Because of this limited complementarity, most *trans*-encoded sRNAs can bind to several target mRNAs (Gottesman, 2005; Prevost *et al.*, 2007). Base-pairing occurs through short and highly conserved regions (Gottesman and Storz, 2011). Most *trans*-encoded sRNAs are 50 – 300 nucleotides in length (Waters and Storz, 2009). These sRNA genes usually contain their own promoter- and terminator sequences and are only expressed under certain physiological conditions i.e. during cell stress or in a virulence-related context. In contrast to *cis*-encoded sRNAs, almost all of the known *trans*-encoded sRNAs require Hfq for function (see 1.2.5). Several different outcomes of sRNA-mRNA base pairing are known so far.

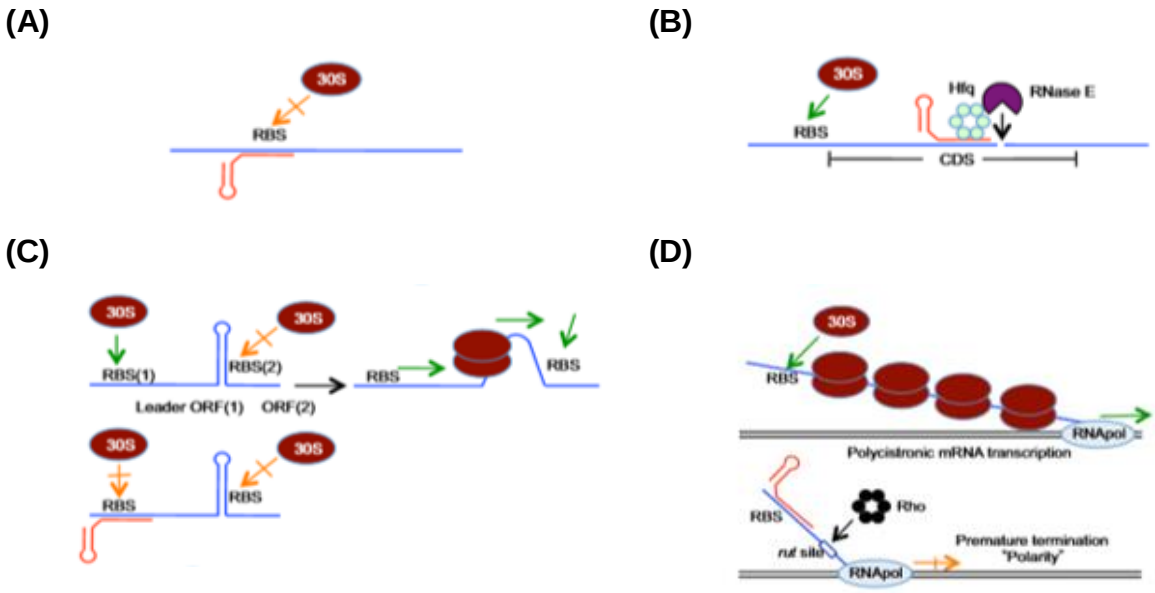
Direct translational inhibition: The majority of *trans*-encoded base pairing sRNAs in Bacteria target translational initiation, as this represents the rate-limiting step during protein synthesis (Kozak, 1999). They act by preventing ribosome-binding and/or decreasing mRNA stability, as exemplified for the *E. coli* sRNA MicA, which acts in concert with Hfq (Figure 7A) (Udekwu *et al.*, 2005). Most inhibitory sRNAs are rapidly degraded concomitant with the mRNA. For instance, binding of the sRNA SgrS to the *E. coli ptsG* mRNA leads to RNase E recruitment, followed by mRNA degradation (Figure 7G) (Vanderpool and Gottesman, 2004).

Indirect translational inhibition: In the case of the *E. coli* sRNA IstR-1, the ribosome-binding site is not directly accessible and translation initiation requires a ribosome standby site about 100 nt upstream of the RBS. The RBS itself lies within a secondary structure that is presumably resolved by transient breathing upon 30S-binding to the standby site. If the sRNA binds within this standby region, translation is inhibited (Figure 7I) (Darfeuille *et al.*, 2007). The *E. coli* sRNA RyhB is transcribed during iron depletion and down-regulates the mRNA encoding the ferric uptake regulator Fur. The *fur* mRNA is translationally

coupled to an upstream open reading frame (*uof*), which is targeted by RyhB. The sRNA binds to the RBS of *uof* and prevents translation initiation. As translation of *fur* is positively coupled to *uof*, *fur* mRNA translation is impeded (Figure 7C) (Vecerek *et al.*, 2007). In *Salmonella*, the sRNA ChiX affects a whole operon. ChiX binds to the RBS of its target *chiP* during transcription, which hinders ribosome-binding to the polycistronic mRNA. Hence, Rho utilization sites (rut) are not shielded by polysomes and premature termination occurs, which prevents transcription from the *chiPQ* operon (Figure 7D) (Bossi *et al.*, 2012).

Direct translational activation: A positive regulation of translation can occur *via* an anti-antisense mechanism. In most cases, the RBS of the target mRNA is unaccessible due to an inhibitory intramolecular secondary structure and the interaction with the sRNA leads to structural remodeling of the mRNA (Morfeldt *et al.*, 1995; Majdalani *et al.*, 2005; Prevost *et al.*, 2007). For instance, the *E. coli* sRNA DsrA binds near the *rpoS* ribosome-binding site, which resolves the secondary structure that interferes with translation (Figure 7E) (Majdalani *et al.*, 1998).

Indirect translational activation: A translational coupling mechanism, analogous to that of RyhB, can also result in positive regulation. This mechanism is employed by the *P. aeruginosa* sRNA PhrS (see Figure 8; 1.3.1.1) (Sonnleitner *et al.*, 2011). Another elaborate way of positive regulation is target mimicry. This mechanism is similar to those observed for protein-binding sRNAs (Figure 6B). In *Salmonella,* the outer membrane porin ChiP is not needed at low chitooligosaccharide concentrations. Therefore, silencing occurs *via* the sRNA ChiX, which binds to the RBS of *chiP* (Figure 7D). If chitobiose is abundant in the medium, the intergenic region (ICR) *chbBC* acts like a decoy for the ChiX sRNA and thereby prevents ChiX-mediated *chiPQ* down-regulation (Figure 7J) (Figueroa-Bossi *et al.*, 2009).



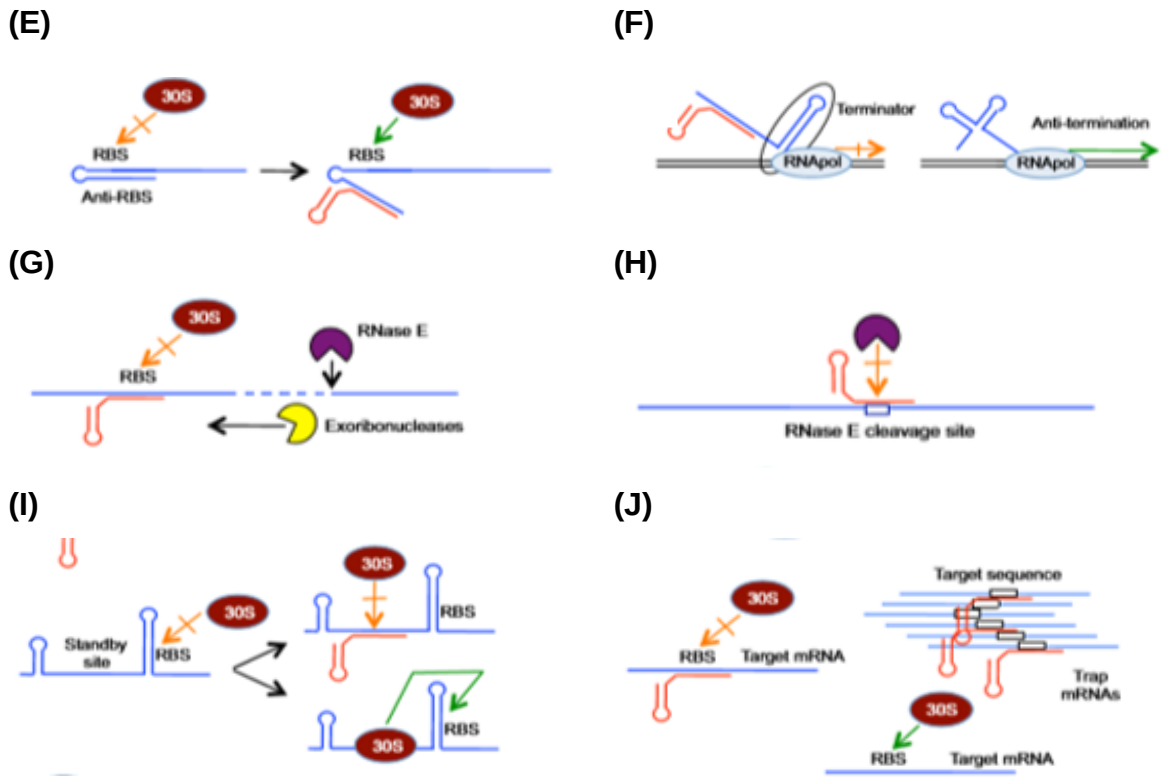


Figure 7. Regulatory mechanisms of base pairing sRNAs.

(A) The binding of a sRNA to the RBS hinders translation initiation (e.g. MicA) (Udekwu *et al.*, 2005). **(B)** A "decay only" mechanism: a sRNA binds, associated with Hfq, in the coding sequence (CDS) of its target mRNA and favors RNase E-dependent cleavage, without inhibiting translation, respectively (e.g. MicC) (Pfeiffer *et al.*, 2009; Bandyra *et al.*, 2012). **(C)** The RBS (RBS(2)) is not accessible because of a secondary structure. Translation only occurs due to translational coupling to an upstream leader ORF sequence (ORF(1)). Binding of a sRNA to the RBS (RBS(1)) of this leader ORF sequence results in translational inhibition, which also affects ORF(2) (e.g. RyhB) (Vecerek *et al.*, 2007). **(D)** Transcriptional polarity: the sRNA cotranscriptionally binds to the RBS of a polycistronic mRNA and the whole operon becomes devoid of ribosomes. A normally unaccessible Rho-binding site is revealed (*rut*), leading to premature termination (e.g. ChiX) (Figueroa-Bossi *et al.*, 2009). **(E)** Activation of translation *via* anti-antisense: the RBS is unaccessible due to a secondary structure that prevents ribosome binding. Binding of a sRNA to the Anti-RBS resolves the secondary structure and translation initiation can proceed (e.g. DsrA) (Majdalani *et al.*, 1998). **(F)** Small RNA-binding results in premature transcriptional termination (e.g. RnaG) (Giangrossi *et al.*, 2010). **(G)** Translational inhibition by a sRNA is followed by rapid degradation of the mRNA (e.g. SgrS) (Vanderpool and Gottesman, 2004). **(H)** Stabilization of a target mRNA: the sRNA binds to an RNase E cleavage site and shields from degradation (e.g. RydC) (Frohlich *et al.*, 2013). **(I)** The 30S ribosomal subunit binds to an upstream ribosome standby site and initiation is possible *via* transient breathing that allows access to the RBS. The sRNA binds to this standby site and inhibits ribosome-binding (e.g. IstR-1) (Darfeuille *et al.*, 2007). **(J)** Small RNAs as traps, decoys or sponges: mRNAs that contain an antisense sequence to a sRNA can titrate away the sRNA, antagonizing target mRNA repression (e.g. ChiX/MicM) (Figueroa-Bossi *et al.*, 2009). Small RNAs are shown in red, a negative regulation of translation is indicated by crossed orange arrows, a positive regulation is shown by green arrows. Figure adapted from Wagner and Romby (2015).

Nevertheless, there are still modes of action that cannot be assigned to any of the above categories. For example, the *Salmonella* sRNA MicC binds within the coding sequence (CDS) of its target *ompD*, but surprisingly this does not influence translation at all. MicC, in concert with the RNA-binding protein Hfq, guides and activates RNase E to initiate mRNA degradation (Figure 7B) (Pfeiffer *et al.*, 2009; Bandyra *et al.*, 2012). In addition, mRNAs can be stabilized upon sRNA-binding, which has been shown for the Hfq-dependent sRNA RydC in *Salmonella*. RydC prevents RNase E degradation of an isoform of the *cfa* mRNA (Figure 7H) (Frohlich *et al.*, 2013).

1.3.1 *P. aeruginosa* sRNAs with annotated functions

1.3.1.1 The sRNA PhrS

The PhrS sRNA consists of 213 nucleotides and belongs to the small group of dual-functional *trans*-acting sRNAs that encode a polypeptide. In case of PhrS, the function of the small peptide is yet unknown (Sonnleitner *et al.*, 2008). PhrS synthesis is controlled by the anaerobic regulator ANR, an orthologue of the *E. coli* FNR protein. The transcriptional regulator is inactive at high oxygen concentrations and is only functional during hypoxic conditions (Zimmermann *et al.*, 1991). ANR is primarily responsible for the activation of genes involved in the arginine deiminase pathway and in denitrification, a process where nitrate and nitrite are used as electron acceptors (Haas *et al.*, 1992; Ye *et al.*, 1995). The ANR recognition sequence is located upstream of the -35 region of the *phrS* promoter. Under low oxygen concentrations, ANR is active and *phrS* is expressed. The transcriptional regulator PqsR is an indirect target of the sRNA PhrS. Translation of the *pqsR* mRNA is coupled to an open reading frame (*uof*), which is located immediately upstream of the *pqsR* RBS. Translation of *uof* is required for PqsR synthesis (Sonnleitner *et al.*, 2011). Under aerobic conditions, the Shine-Dalgarno sequence of *uof* is sequestered by a secondary structure and only low translation of both *uof* and *pqsR* occurs. Upon activation of ANR during hypoxic conditions, *phrS* is transcribed. PhrS binds to and thereby resolves the secondary structure in the *uof* RBS, leading to increased *uof* translation that subsequently activates *pqsR* translation by positive translational coupling (Figure 8) (Sonnleitner *et al.*, 2011). PqsR is part of the QS hierarchy: it is involved in virulence factor production and in the biosynthesis of HAQs, like PQS and its precursor HHQ (Deziel *et al.*, 2005). PhrS indirectly enhances PQS production and pyocyanin synthesis *via* PqsR (Sonnleitner *et al.*, 2011).

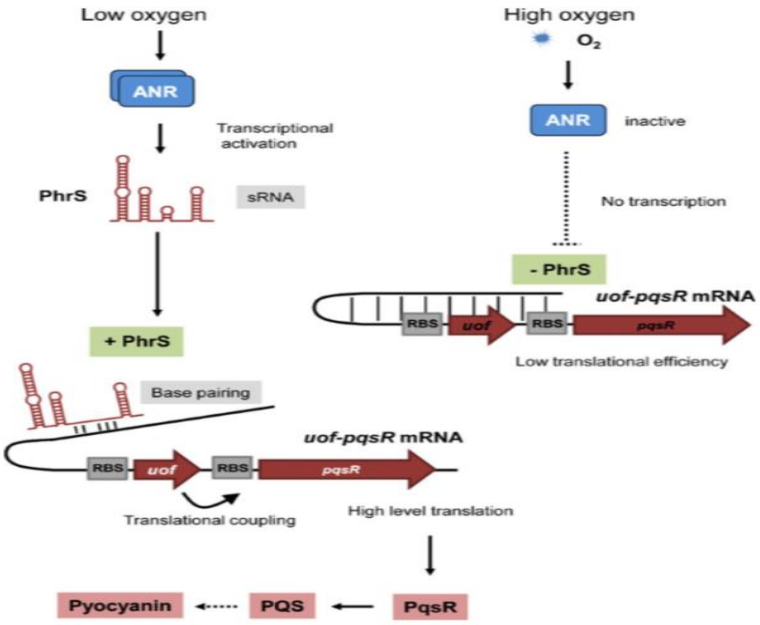


Figure 8. The sRNA PhrS indirectly enhances PqsR synthesis.
The transcriptional regulator ANR controls transcription of *phrS*. PhrS base pairs with the upstream region of a small open reading frame (*uof*) that is positively translationally coupled to the *pqsR* mRNA. Binding of PhrS increases the accessibility of the *uof* RBS and therefore enhances translation of *uof* and, as a consequence, that of *pqsR (mvfR).* The transcriptional regulator PqsR positively regulates PQS production and many genes involved in pyocyanin synthesis. Figure taken from Sonnleitner and Haas (2011).

1.3.1.2 The sRNA CrcZ

CrcZ is a 407 nt long RNA, which is transcribed from an RpoN-dependent promoter. The response regulator CbrB is required for transcriptional activation of *crcZ* (Sonnleitner *et al.*, 2009; Abdou *et al.*, 2011). CrcZ is able to sequester Hfq *via* several binding sites. The first 151 nucleotides of the CrcZ sRNA contain three experimentally verified A-rich Hfq-binding sites (Sonnleitner and Bläsi, 2014). The mechanism of action resembles the titration of RsmA by the RNAs RsmY and RsmZ (see 1.3.1.3). The binding of CrcZ to Hfq occurs at the distal side of the protein (Sonnleitner and Bläsi, 2014). Both CrcZ and mRNA bind to the distal face of Hfq and can compete for binding (Figure 9). Hfq functions as a direct translational repressor of several genes encoding functions involved in the degradation of alternative carbon sources (e.g. *amiE*, *estA* and *phzM*). Carbon catabolite repression (CCR) ensures that “good carbon sources”, like succinate, are used preferentially, and only when they are depleted, the degradation of less favorable compounds ensues (Smyth and Clarke, 1975; Collier *et al.*, 1996). Therefore, CCR prevents translation of mRNAs needed for the degradation of poor carbon sources. Hfq acts as a translational repressor and interferes with translation initiation of these mRNAs. During this

favorable conditions, *crcZ* is transcribed at low levels. When the favored carbon compound is depleted, *crcZ* transcription increases as a consequence of activation *via* the CbrA/B two-component system. Subsequently, Hfq is titrated by CrcZ and the repressive function on CCR-regulated genes is alleviated (Figure 9) (Sonnleitner and Bläsi, 2014).

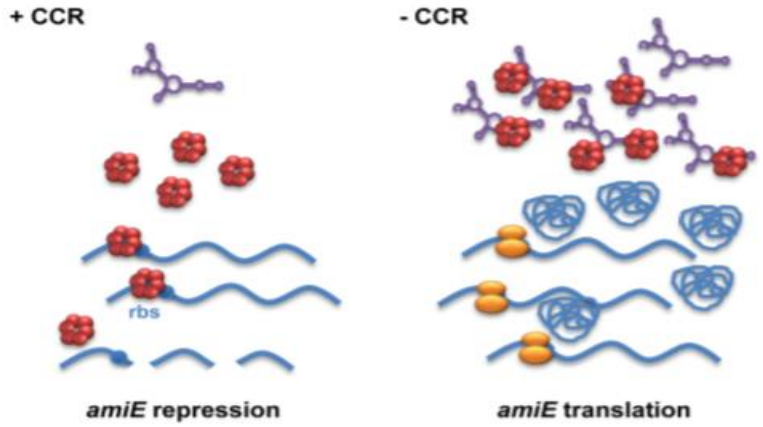


Figure 9. The sRNA CrcZ regulates carbon catabolite repression.
In the presence of a preferred carbon compound (i.e. succinate), the CrcZ levels are low. Hfq binds to the RBS of target mRNAs (i.e. *amiE*) and inhibits translation (+ CCR). Upon growth in a poor carbon source, like acetamide, CCR-regulated genes are not repressed (- CCR). Transcription of *crcZ* is activated by the CbrA/B two-component system. High levels of CrcZ titrate Hfq, which alleviates the repression of *amiE* translation. Purple, CrcZ RNA; red hexamer, Hfq; blue lines, mRNA; yellow, ribosomes. Figure adapted from Sonnleitner and Bläsi (2014).

1.3.1.3 The sRNAs RsmY/Z

The response regulator GacA activates expression of two regulatory RNAs, termed RsmY and RsmZ. They sequester the RNA-binding protein RsmA, and therefore antagonize its effect on translation (Figure 3 and Figure 10) (Kay *et al.*, 2006; Brencic *et al.*, 2009). These sRNAs belong to the CsrB/RsmY family with several functional homologues in other Pseudomonads and enteric bacteria (Romeo *et al.*, 2013). They both contain a palindromic UAS, which was identified as the putative GacA-binding site (Kay *et al.*, 2006). GGA motifs are necessary for the RsmA-binding properties of RsmY in *P. fluorescens* (Valverde *et al.*, 2004). These motifs are also present in the *P. aeruginosa* orthologue. RsmY is 124 nucleotides in length and contains six GGA motifs (Kay *et al.*, 2006). Sorger-Domenigg *et al.* (2007) revealed that Hfq binds to RsmY and increases the stability of the sRNA. It was shown by Sonnleitner *et al.* (2006), that the RsmY steady-state levels are reduced by 50% in a PAO1*hfq*- strain. The sRNA RsmZ also possesses six GGA motifs over a length of 125 nucleotides (Heurlier *et al.*, 2004; Burrowes *et al.*, 2005). In contrast to RsmY, RsmZ does not interact with Hfq (Sorger-Domenigg *et al.*, 2007).

In addition to the transcriptional activation by GacA, *rsmY* expression is influenced by the HptB histidine phosphotransfer protein. HptB is phosphorylated by three hybrid sensor kinases (Figure 10) and negatively regulates the phosphorylation activity of the response regulator PA3346. As a consequence, PA3347 stays unphoshorylated and can bind to an uncharacterized anti-sigma factor. This triggers the release of an unknown sigma factor that may negatively regulate *rsmY* expression (Hsu *et al.*, 2008; Bordi *et al.*, 2010). Furthermore, in a biofilm environment, RNase G, which is regulated by the BfiSR two-component system, is involved in RsmZ, but not RsmY degradation (Figure 10) (Petrova and Sauer, 2010).

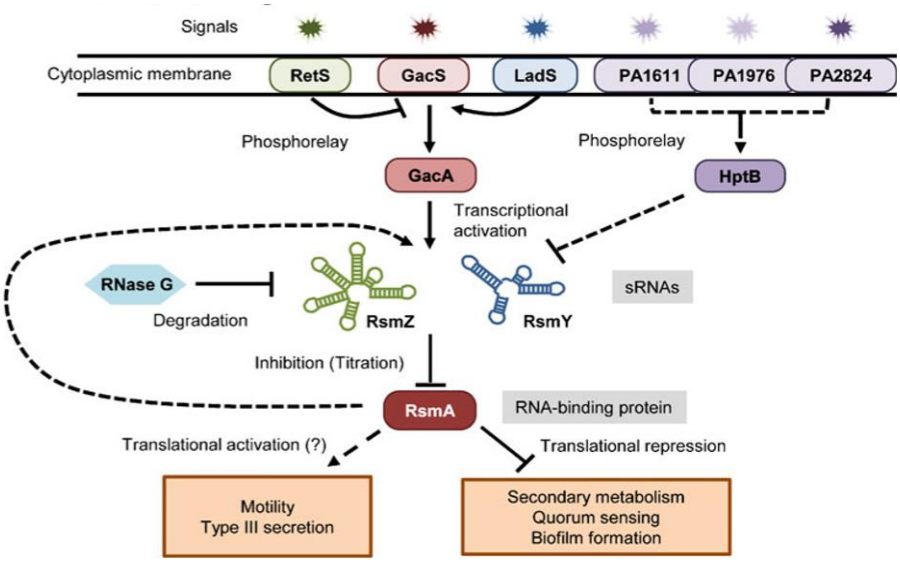


Figure 10. Model of the Gac/Rsm regulatory network in *P. aeruginosa.*

The sensor kinase GacS phosphorylates and activates the response regulator GacA upon receiving a hitherto unknown signal. GacA mediates transcriptional activation of the RNAs RsmY and RsmZ, which in turn can bind to the post-transcriptional regulator RsmA. As a result, RsmA-mediated translational repression is relieved. RsmA negatively impacts on biofilm formation, secondary metabolism and quorum sensing. Further explanations are given in the text. Arrows, positive regulation; orthogonal lines, negative regulation; continuous lines, direct control; dotted lines, indirect control. Adapted from Sonnleitner and Haas (2011).

1.3.2 Non-characterized *P. aeruginosa* sRNAs

In *P. aeruginosa*, the amount of characterized sRNAs with assigned functions is considerably low. Many orphan sRNAs have been identified in the past using biocomputational (Livny *et al.*, 2006; Gonzalez *et al.*, 2008; Sonnleitner *et al.*, 2008) or deep sequencing (Gómez-Lozano *et al.*, 2012; Ferrara *et al.*, 2012) approaches. It remains a major challenge to decipher their functions, especially with regard to virulence and antibiotic resistance. A biocomputational search for *trans*-encoded sRNAs performed by Sorger-

Domenigg (2010) in *P. aeruginosa,* revealed two new sRNAs. The RNA transcripts PaeII and PaeIII fulfilled the following criteria. The corresponding genes contain a promoter region, a ρ-independent terminator and the transcripts have a length ranging between 50 - 300 nucleotides. Moreover, both sRNAs are located in intergenic regions.

1.3.2.1 PaeII

The sRNA PaeII is located in the intergenic region between the two hypothetical proteins PA4040 and PA4041 (Winsor *et al.*, 2016). PaeII was predicted to contain three stem-loop structures (Figure 11) and is 108 nucleotides in length. The sRNA is processed by RNase E, resulting in a 76 nt fragment (Figure 11; the cleavage site is indicated by an arrow) (Sorger-Domenigg, 2010). The presence of this fragment was verified by RNAseq (transcriptome profiling) (Rindler, 2014). A BLASTN 2.3.1+ (Zhang *et al.*, 2000) search confirmed that this 3'-terminal truncated version is conserved in *P. aeruginosa strains* and *P. stutzeri, P. alcaligenes, P. pseudoalcaligenes, P. balearica, P.sp. L10.10, P. agarici and P. fragi. PaeII* is highly expressed in minimal medium but poorly in LB medium. Basal *paeII* levels are transcribed from a σ^{70}-dependent promoter, but full expression is σ^{54}-dependent. However, PaeII is not required for growth in minimal medium, since a PAO1Δ*paeII* strain is not impaired in growth in BM2 (Sorger-Domenigg, 2010) or M9 medium. The high PaeII levels upon growth in minimal medium suggested a link to the stringent response. RelA and SpoT regulate the levels of ppGpp in the cell (Chatterji and Ojha, 2001). This alarmone indirectly activates genes that require alternative sigma factors for their transcription (Magnusson *et al.*, 2005). Therefore, the reduction of the *paeII* levels in a *relA spoT* double mutant were in line with the observed σ^{54}-dependent expression.

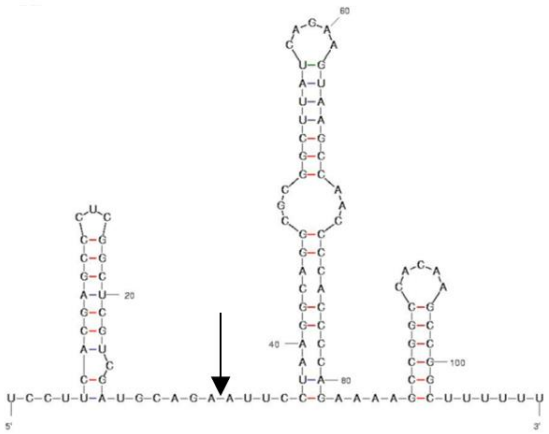


Figure 11. Structure prediction of the sRNA PaeII.

PaeII secondary structure prediction with mfold (Zuker, 2003). The RNAse E cleavage site between nucleotide 32 and 33 in the single-stranded AU-rich region is indicated by an arrow. Figure adapted from Sorger-Domenigg (2010).

Hfq has distinct functions in post-transcriptional regulation and RNA stability, in most cases concomitant with sRNA-mediated regulation (Vogel and Luisi, 2011). Sorger-Domenigg (2010) determined the PaeII steady state levels by Northern-blotting and showed that the PaeII levels were decreased in a PAO1*hfq*- strain, but the stability of the sRNA was not reduced in the absence of Hfq (Sorger-Domenigg, 2010).

A two-dimensional gel analysis, conducted with strains grown in BM2 medium supplemented with 20 mM mannitol, revealed that four proteins were differentially abundant in a PAO1Δ*paeII* strain when compared with the wild-type strain (Sorger-Domenigg, 2010). PA4315 is the RNA-binding protein MvaT, which controls pyocyanin metabolism (Li *et al.*, 2009), but the function of the three other proteins is yet unknown. Although MvaT levels were reduced in the *paeII* deletion strain, pyocyanin synthesis was indistinguishable from the wild-type strain (Sorger-Domenigg, 2010).

Microarray data revealed a surprisingly small number of differentially expressed genes upon *paeII* deletion in BM2 medium supplemented with 20 mM mannitol (Sorger-Domenigg, 2010). When compared with the wild-type strain, two genes were found to be up-regulated and seven were down-regulated in the *paeII* mutant. Interestingly, five of the genes with reduced expression levels form an operon (PA3515 - PA3519) (Winsor *et al.*, 2016) and the other two are directly adjacent (PA3520 and PA3523). The functions of the genes within this operon is unknown, as well as the one of PA3520. However, PA3523 is involved in the transport of small molecules (Winsor *et al.*, 2016). The two genes up-regulated upon *paeII* deletion were *sdsA1*, an SDS hydrolase, and the L-lactate permease gene *lldP* (Winsor *et al.*, 2016). However, *sdsA1::lacZ* translation was comparable in the PAO1Δ*paeII* strain and the wild-type strain (E. Sonnleitner, unpublished data). Another transcriptome analysis, based on RNA-sequencing, was carried out by Rindler (2014) with strains grown in BM2 medium supplemented with 20 mM succinate. Rindler (2014) changed the carbon source used by Sorger-Domenigg (2010), because mannitol is a poor carbon source for *P. aeruginosa*, which results in a substantially reduced growth rate. In addition, a Northern-blot confirmed that the PaeII levels are only slightly elevated upon growth in mannitol when compared to succinate (Sorger-Domenigg, 2010). In this RNA-seq experiment, transmembrane transport and Zur-regulated genes were enhanced upon *paeII* over-expression when compared with a *paeII* deletion strain. Strikingly, many of these transport genes were found to be associated with Zn^{2+} uptake (Pederick *et al.*, 2015). Out of these, four TonB-dependent outer membrane protein genes were up-regulated upon *paeII* over-expression: PA0781 (*znuD*; 10.2-fold), PA1922 (*cirA*; *17-fold),* PA2911 (2.8-fold) and PA4837 (14.3-fold). Additionally, three zinc-specific solute-binding proteins (SBPs) showed enhanced transcription levels in the presence of PaeII: PA2913 (2.2-fold), PA4063 (4.7-fold), PA4066 (2.9-fold). Apart from the ZnuABC import system (Figure 4), two other potential periplasmic zinc importer systems have been described:

PA2912/PA2914 and PA4064/PA4065 (Pederick *et al.*, 2015). Either operon was up-regulated upon *paeII* over-expression. In addition, Zn^{2+} import into the cytoplasm is mediated by an alternative system using a P-type ATPase (PA2435/*hmtA*; 1.9-fold up-regulated) (Lewinson *et al.*, 2009). Moreover, many zinc-independent paralogs of zinc-requiring genes were up-regulated upon *paeII* over-expression: *dksA2* (PA5536; 12.3-fold), which encodes a variant of the DNA-binding protein DksA (1.2.3) (Blaby-Haas *et al.*, 2011), *pyrQ* (PA5541; 33.1-fold) a dihydroorotase involved in pyrimidine synthesis (Brichta *et al.*, 2004) and *folE2* (PA5539; 14.7-fold), which is a putative GTP cyclohydrolase (Sankaran *et al.*, 2009). Ribosomal proteins also have variants that do not contain metal ion residues (Makarova *et al.*, 2001). This ensures that ribosome function is independent of cellular metal ion limitations. Two zinc-independent forms of 50S ribosomal proteins were up-regulated in the presence of PaeII: L36 (PA3600; 9.6-fold) and L31 (PA3601; 8.1-fold). All of these genes were likewise up-regulated in a *znuA* mutant of PAO1 more than 4-fold (Pederick *et al.*, 2015). Despite of these indications that PaeII might be involved in zinc homeostasis, no direct targets of this sRNA have been identified so far.

1.3.2.2 PaeIII

The *trans*-encoded sRNA PaeIII is 98 nucleotides in length (Figure 12) and located in the intergenic region flanked by PA3535 and PA3536 (Winsor *et al.*, 2016). PaeIII is transcribed from a σ^{70}-dependent promoter. According to a BLASTN 2.3.1+ (Zhang *et al.*, 2000) search, PaeIII is only conserved in *P. aeruginosa* strains and not in other Pseudomonads. This sRNA is expressed in rich medium (LB), particularly in stationary phase, under pH stress and at elevated temperatures. Despite of this, *paeIII* expression does not depend on the stationary and stress sigma factor RpoS (Sorger-Domenigg, 2010; Rindler, 2014). In addition, the PaeIII levels were found to be 2-fold enhanced in an *rsmA* mutant strain. As opposed to PaeII, the levels of the sRNA PaeIII were increased in an *hfq*- strain when compared with the wild-type PAO1 strain. Besides, PaeIII binds to Hfq (Kd ~ 100 nM), but PaeIII stability does not depend on Hfq (Sorger-Domenigg, 2010).

A proteomics approach revealed that the abundance of PA4739, a hypothetical protein, and PurE, which is an enzyme involved in purine biosynthesis, was reduced upon *paeIII* over-expression (Sorger-Domenigg, 2010). The levels of PA1202, a protein of unknown function, were enhanced in this strain. A putative effect of PaeIII on PurE and PA1202 was further investigated with translational reporter gene fusions. However, no differences concerning the β-galactosidase activity were detected for both fusions, when a *paeIII* over-expression strain was compared with the wild-type strain.

A Northern-blot performed by Sorger-Domenigg (2010), showed that the PaeIII levels were enhanced upon exposure to human serum, synthetic sputum cystic fibrosis medium

(SCFM) and in the absence of RpoN. A microarray analysis indicated that genes required for anaerobic respiration and pyocin production were down-regulated in a *paeIII* over-expression strain (Sorger-Domenigg, 2010). Moreover, the gene *mexX* displayed 7.3-fold higher transcript levels when compared with the wild-type strain. MexX is part of the MexXY multidrug efflux system, which contributes to the resistance to aminoglycoside antibiotics in *P. aeruginosa* (Mine *et al.*, 1999; Morita *et al.*, 2012). This led to the assumption that PaeIII might enhance the resistance to this class of antibiotics. Therefore, Sorger-Domenigg (2010) compared the resistance against tetracycline of a *paeIII* over-expression strain, a *paeIII* deletion mutant and the wild-type strain. The *paeIII* over-expression strain was less susceptible to tetracycline, while the *paeIII* mutant showed increased susceptibility when compared with the wild-type strain. Sorger-Domenigg (2010) suggested that PaeIII might influence *mexX* levels by interacting with a regulator of this operon. MexZ is one of the transcriptional regulators that negatively influence *mexXY* expression (Matsuo *et al.*, 2004). Subsequently, the β-galactosidase values conferred by transcriptional and translational fusions of *mexZ* to the *lacZ* reporter gene were determined. However, the MexZ-LacZ synthesis was not altered upon *paeIII* over-expression when compared with the wild-type strain. ArmZ (PA4571) is an anti-repressor of MexZ activity (Hay *et al.*, 2013). The gene encoding ArmZ was 4.6-fold up-regulated upon *paeIII* over-expression. However, the resulting β-galactosidase values from transcriptional and translational *armZ-lacZ* fusions were similar to those in the wild-type strain (Sorger-Domenigg, 2010). The gene PA0547, a probable transcriptional regulator, which was reported to increase aminoglycoside resistance (Struble and Gill, 2009), was up-regulated upon *paeIII* over-expression (2.1-fold). Sorger-Domenigg (2010) identified a possible interaction of PaeIII and PA0547 with the program TargetRNA (Tjaden, 2008). Nevertheless, no further analysis was performed to investigate if PA0547 is a direct target of PaeIII.

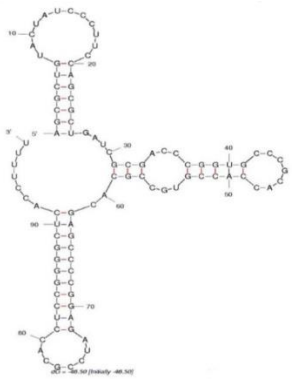


Figure 12. Secondary structure of PaeIII.

Secondary structure of the sRNA PaeIII, which is 98 nucleotides in length. The structure was predicted with the program mfold (Zuker, 2003) and contains two stem-loops and a strong ρ-independent terminator. Figure adapted from Sorger-Domenigg (2010).

The gene encoding the alternative sigma factor RpoS was 2.9-fold down-regulated upon *paeIII* over-expression (Sorger-Domenigg, 2010). A search using the program TargetRNA (Tjaden, 2008) identified a potential interaction site including the RBS and the start codon of *rpoS*. The β-galactosidase values conferred by a plasmid encoded translational *rpoS::lacZ* fusion were in line with the transcriptome data. A *paeIII* mutant strain produced 2-fold higher β-galactosidase activity in stationary phase when compared with the wild-type strain. Rindler (2014) further analyzed *rpoS* translation with reporter gene fusions. *RpoS::lacZ* translation was reduced upon pulse expression of *paeIII* when compared with the wild-type strain. In addition, mutations in the putative interaction site of PaeIII with the *rpoS* mRNA abrogated this effect. Subsequently, compensatory mutants of *paeIII* and the 5' UTR of the *rpoS* mRNA were constructed. However, it was not possible to verify a direct interaction between PaeIII and the *rpoS* mRNA (Rindler, 2014). Hence, up to now no direct targets or regulatory mechanisms concerning PaeIII have been revealed.

1.4 Aim of the work

The aim of this work was to elucidate the function of the *P. aeruginosa* sRNAs PaeII and PaeIII. Several hypotheses on the involvement of PaeII and PaeIII in physiological processes, based on previous transcriptome data, were experimentally tested. Initial experiments revealed that PaeII is highly expressed in BM2 medium and *paeII* transcription is to a great extent σ^{54}-dependent. Therefore, a putative role of PaeII during nitrogen limitation was assessed. A second aim was to investigate if the mRNA encoding the transcriptional regulator DksA2 is a direct target of PaeII.

PaeIII, on the other hand, is expressed in LB medium and reaches the highest levels at the onset of stationary phase. The first objective was to investigate a possible role of PaeIII during growth in stationary phase and at elevated temparatures. In addition, *ladS* and *rpoS*, two putative direct targets, should be verified.

2 Materials

2.1 Strains

2.1.1 *E. coli*

Table 2. *E. coli* strains.

Strain	Genotype	Source
TOP10	F$^-$, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), (Φ80*lacZ*ΔM15), Δ*lacX74*, *deoR*, *recA1*, *araD*139Δ(ara-leu)7697, *galK*, *rpsL*(StrR), *endA1, nupG*	Stratagene
DH5α	*recA1 endA1 hsdR17 thi-1 supE44 gyrA96 relA1 deoR* Δ(*lacZYA-argF*) *U169* (Φ80*lacZ*ΔM15)	Sambrook *et al.* (2001)

2.1.2 *P. aeruginosa*

Table 3. *P. aeruginosa* strains.

Strain	Genotype	Source
PAO1	wild-type	Holloway *et al.* (1979)
PAO1Δ*paeI*	*paeI* in-frame deletion	Sorger-Domenigg (2010)
PAO1Δ*paeIII*	*paeIII* in-frame deletion	Sorger-Domenigg (2010)
PAO6358	Δ*rpoN*	Heurlier *et al.* (2003)
PAO6743	*ntrC*::Gm^R	Haas Lab
PAO6711	Δ*cbrB*	Sonnleitner *et al.* (2009)
PAO6281	*gacA*::SpSm	Reimann *et al.* (1997)
PAO1PAZHI3	Δ*rsmA*	Pessi *et al.* (2001)
PAO1*rpoS*$^-$	*rpoS101*::*aacCI*, Gm^R	Suh *et al.* (1999)
PAO1Δ*rsmY*	*rsmY*::*aacCI*, Gm^R	Sonnleitner *et al.* (2006)

2.2 Plasmids

Table 4. Plasmids.

Plasmid	Features	Source
pME4510	Broad-host-range promoter-probe plasmid, Gm^R	Rist *et al.* (1998)
pME4510*paeIII*	pME4510 comprising *paeIII*	Sorger-Domenigg (2010)
pME4510*lacI*QpTA-C*lacO*	Plasmid for inducible expression of sRNAs from the *tac* promoter, Gm^R	Bläsi Lab
pME4510*tacpaeIII*	pME4510*lacI*QTAC*lacO* comprising *paeIII*	This work
pME6015	Cloning vector for translational *lacZ* fusions; Tc^R	Schnider-Keel *et al.* (2000)
pME6016	Cloning vector for transcriptional *lacZ* fusions; Tc^R	Schnider-Keel *et al.* (2000)
pME6015*dksA2::lacZ*	pME6015 comprising the *dksA2::lacZ* fusion gene	Bläsi Lab
pME6016*dksA2-lacZ*	pME6016 comprising the *dksA-lacZ* fusion gene	This work
pME6016*rpoN-lacZ*	pME6016 comprising the *rpoN-lacZ* fusion gene	This work
pJT19	IncP expression vector with inducible Pm promoter; Km^R	Winther-Larsen *et al.* (2000)
pJT*paeII*	pJT comprising *paeII*	Rindler (2014)
pME9806	pME6016 comprising the *crcZ-lacZ* fusion gene	Sonnleitner *et al.* (2009)
pME7233	pME6016 comprising the *phrS-lacZ* fusion gene	Sonnleitner *et al.* (2011)
pME9851	pME6016 comprising the *rpoN::lacZ* fusion gene	Karine Lapouge Lab
pME3843	pME6010 comprising the p*tac-hcnA::lacZ* fusion gene	Pessi *et al.* (2001)

2.3 DNA oligonucleotides

Table 5. DNA oligonucleotides.

Cleavage sites are underlined, T7 promoter sequences are highlighted in bold.

Name	Sequence (5'→ 3' direction)	Cleavage site
O119	GCCCGACGCTGCCTACAT	
N119	GCTTCTTCTCCCGCTCCAG	
L37	ATCGTAGTCCGGATCGCAGT	
M37	AGTCATGAATCACTCCGTGGTA	
I26	CCCCACACTACCATCGGCGATGCGTCG	
V28	GGCTTTTCTGGGGTGGGGTTGGCTT	
W28	CGTGCGGCACGGTGGTGCGGG	
T26	GATTCCCTGAGTTTCC	
M31	GC**TCTAGAT**AATACGACTCACTATAGGCCAGCGCTTCTATCCCTCCAG	*Xba*I
O29	GGAATTCAAAAGGTGAGCCCCGGAGGTGCG	*EcoR*I
A57	**TGCTCTAGACGTAATACGACTCACTATA**GTCCTTCCGAGCCCTCGGC	*Xba*I
L29	GGAATTCGCATTAAAAAAGCCGGCTTGTGG	*EcoR*I
U26	CCGTCTAGACG**TAATACGACTCACTATA**GTCAGGACAGCGCAGAAGC	*Xba*I
V26	AAACTGCAGGAGCGACGCGGTTTTCCTCGGGC	*Pst*I
A121	TTTTTCCCGGGAGCGCTGTACTATCCCTTCCA	*Sma*I
C121	TTTTTCTGCAGAAAAGGTGAGCCCCGGAGGT	*Pst*I
D124	TTTTTGAATTCCTTCGCCGTGCTCA	*EcoR*I
E124	TTTTTCTGCAGCTGGGAAATTTAAAACG	*Pst*I
F124	TTTTTGAATTCATCATCCATCACCTCAAG	*EcoR*I
G124	TTTTTCTGCAGCACCATCCACTACTACGG	*Pst*I

2.4 Antibodies

Table 6. Antibodies for Fluorescent Western-blotting.

Name	Target	Source
Protein-specific		
RpoS anti-rabbit antibody	RpoS	Bläsi Lab
Ribosomal protein S2 anti-rabbit antibody	S2	Bläsi Lab
Ribosomal protein S4 anti-goat antibody	S4	Moll Lab
For detection		
IRDye 800 conjugated anti-rabbit IgG	Rabbit IgG	Rockland Immunochemicals
IRDye 800 conjugated anti-goat IgG	Goat IgG	Rockland Immunochemicals

2.5 Growth conditions

2.5.1 Media

The bacterial cultures were grown in glass flasks at 37°C with shaking (160 rpm), unless stated otherwise. For growth monitoring the optical density at 600 nm (OD_{600}) was measured with a spectrophotometer (Hitachi U-1100).

LB medium	
Peptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

If needed, the medium was solidified with 15 g/L agar.

BM2 medium	
K_2HPO_4	40 mM
KH_2PO_4	22 mM
$(NH_4)_2SO_4$	7 mM
MgSO4	0.5 mM
FeSO4	0.01 mM

20 mM succinate or 20 mM mannitol were added as a carbon source.

M9 medium	
Na_2HPO_4	48 mM
KH_2PO_4	22 mM
NaCl	9 mM
$MgSO_4$	2 mM
$CaCl_2$	0.1 mM
Glucose	15 mM
NH_4Cl	19 mM

2.5.2 Antibiotics

Antibiotics were added to ensure plasmid maintenance at a final concentration of 50 µg/ml gentamicin, 100 µg/ml tetracycline, 400 µg/ml kanamycin for *P. aeruginosa,* and 15 µg/ml gentamicin, 25 µg/ml tetracycline, 50 µg/ml kanamycin for *E. coli*.

2.6 Buffers and solutions

2.6.1 RNA isolation

RNA protection solution	
Roti-Aqua-Phenol (Roth) in 96% EtOH	5%

Lysis buffer	
SDS	2%
Na-acetate	30 mM
EDTA	3 mM

Adjust to pH 5.5

DEPC-treated water
1 ml DEPC (Roth) was added to 1L ddH_2O, the bottle was shaken vigorously and left overnight before autoclaving.

2.6.2 Northern-blot analysis

Washing buffer I (1L)	
20X SSC	100 ml
SDS	0.1%

Washing buffer II (1L)	
20X SSC	5 ml
SDS	0.1%

8% PAA/8M Urea gel (40 ml)	
Urea	19.2 g
10X TBE	4 ml
40% polyacrylamide (Rotiphorese Gel 40)	8 ml

For a single 8% PAA/8M Urea gel (5 ml) 50 µl 10% APS and 5 µl TEMED were added.

Hybridization solution (250 ml)	
BSA	0.04%
PVP	0.04%
Ficoll	0.04%
EDTA	10 mM
20X SSC	62.5 ml
SDS	0.2%

The hybridization solution was prepared with DEPC-treated water. Before use, 2.5 g Dextran 500 (Roth) and 250 µl herring sperm DNA (10 mg/ml; pre-heated at 95°C for 10 min.), were added.

20XSSC	
NaCl	3 M
$C_6H_5Na_3O_7$	344 mM

Adjust to pH 7 with NaOH.

10X TBE	
Tris Base	892 mM
Boric acid	890 mM
EDTA pH 8	20 mM

Adjust to pH 8 with boric acid.

RNA loading dye (1 ml)	
Formamide	940 µl
10X TBE	40 µl
Xylene cyanol	2%
Bromphenol blue	2%

2.6.3 Transformation

Glycerol/MOPS solution	
Glycerol	15%
MOPS	1 mM

2.6.4 Western-blot analysis

SDS PAGE Running gel 12%	
ddH_2O	2.1 ml
Lower-Tris	1.5 ml
30% Acrylamid	2.4 ml
10% APS	14.4 µl
TEMED	7.2 µl

SDS PAGE Stacking gel	
ddH_2O	1.5 ml
Upper-Tris	0.6 ml
30% Acrylamid	270 µl
10% APS	10 µl
TEMED	20 µl

Lower-Tris	
Tris	1.5 M
SDS	0.4%

Adjust to pH 8.8 with HCl.

Upper-Tris	
Tris	0.5 M
SDS	0.4%

Adjust to pH 6.8 with HCl.

10X TBS buffer	
NaCl	1.37 M
KCl	27 mM
Tris	248 mM

Adjust to pH 7.5 with HCl.

Running buffer	
Tris	25 mM
Glycine	200 mM
SDS	0.1%

Transfer buffer	
Glycin	39 mM
Tris	48 mM
SDS	0.037%
Methanol	20%

Adjust to pH 8.3

2X Laemmli buffer (20 ml)	
Tris/HCl (pH 6.8)	250 mM
SDS	6%
Glycerol	20%
β-mercaptoethanol	10%
Bromophenol blue	0.02%

TBST buffer	
Tween in 1x TBS	0.2%

Blocking solution (TBSM)	
Skimmed milk powder in 1x TBS	5%

Stripping buffer	
SDS	1%
Glycine	100 mM

Adjust to pH 2.8

2.6.5 β-Galactosidase assay

Z-buffer	
Na_2HPO_4	60 mM
$NaH_2PO_4 * 2\ H_2O$	40 mM
KCl	10 mM
$MgSO_4 * 7\ H_2O$	1 mM

270 µl β-mercaptoethanol were added per 100 ml before use.

3 Methods

3.1 RNA isolation

Total RNA was isolated using the hot-phenol extraction method described by Leoni *et al.* (1996). The cells were grown under the respective conditions and 1 ml of culture was treated with 125 µl RNA protection solution (2.6.1) before centrifugation (13,000 rpm, 2 min., 4°C). The pellet was resuspended in 50 µl DEPC-treated water and then 750 µl pre-heated (65°C) lysis-phenol buffer (2.6.1) were added. The aqueous phase was obtained by phenol-chloroform extraction after vigorous vortexing for several minutes. The nucleic acids were precipitated overnight at -20°C in 0.1 volume 3 M sodium acetate and 2.5 volumes 96% ethanol. After centrifugation (16,400 rpm, 30 min., 4°C) and washing with 70% ethanol, the nucleic acids were treated with DNase I (recombinant, RNase-free DNase I (Roche)) for 60 minutes at 37°C. The RNA was purified twice by phenol-chloroform extraction and ethanol precipitation, before resuspension in 20 µl DEPC-treated water. The RNA concentration was measured using a Nano Drop ND-1000 spectrophotometer (PEQLAB). The RNA purity was determined by the ratio of the optical density at 260 and 280 nm.

3.2 Real-time quantitative PCR

The RNA was isolated as afore-mentioned (3.1). After three rounds of phenol-chloroform extraction and ethanol precipitation, no DNA contamination could be detected by PCR with primers L37 and M37 (Table 5), amplifying a 192 nt fragment of the 16S rRNA gene. 200 ng RNA were used to synthesize cDNA with 20 Units Promega AMV Reverse Transcriptase and commercially available random hexamer primers (0.1µg/µl; Promega). RNA and random hexamers were heated to 70°C for 5 minutes, before adding 6 µl DEPC-treated water, 2 µl dNTP Mix (10 µM; Promega), 4 µl 5X AMV buffer and 2 µl AMV-RT (20 units). The reaction was incubated at 37°C for 1 h. The RT-qPCR reactions were performed with the Eppendorf MasterCycler RealPlex system. One reaction contained 4 µl HOT FIREPol® EvaGreen® qPCR Mix Plus, 0.5 µl (10 µM) primers N119 and O119 (amplification of a 203 nt fragment of the *dksA2* gene (Table 5)), 20 ng cDNA and 10 µl DEPC-treated water. The 20 µl volume reactions were amplified using the following program: 94°C / 15 min, then 40 cycles at 94°C / 20 sec, 58°C / 20 sec and 72°C / 20 sec, followed by 94°C / 15 sec. Subsequently, a melting curve analysis was carried out to exclude unspecific amplifications (45°C / 15 sec up to 95°C / 15 sec within 20 min). The RT-qPCR reactions were performed in triplicate for each sample, and for every strain

three biological replicates were used. In addition, no-template controls and reactions devoid of reverse transcriptase were included. Fold changes were calculated with the comparative C_T method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008). A PCR fragment of the 16S rRNA amplified by the primers L37 and M37 (Table 5) was used as an endogenous control for normalization.

3.3 Northern-blot analysis

Total RNA was isolated as described in 3.1 and the concentration was determined using a Nano Drop ND-1000 spectrophotometer (PEQLAB). Unless indicated otherwise, 4 µg total RNA were used for Northern-blot analysis. The samples were mixed 1:1 with RNA loading dye (2.6.2) and heated at 65°C for 5 minutes, before separation on a denaturating 8% polyacrylamide 8 M urea gel (2.6.2). The nucleic acids were bound to an Amersham Hybond-N+ nylon membrane (GE Healthcare Life Sciences) (45 min. at 12 V) by semi-dry electrophoretic transfer. Immobilization was accomplished by autocrosslinking twice with UV light (from 1200 to 0 µJoules). The membrane was pre-hybridized with hybridization solution (2.6.2) at 55°C for 2 h in a glass tube, followed by hybridization with gene-specific ^{32}P 5'- end labelled oligonucleotides (V28, PaeII; W28, PaeIII or T28, RsmY, Table 5) overnight at 55°C. Thereafter, the membrane was washed with 20 ml washing buffer I at 55°C for 10 minutes, followed by the same washing procedure with washing buffer II (2.6.2). The blot was rinsed shortly with water before exposure to a storage phosphor screen overnight. Signals were detected with a phosphorimager (Typhoon FLA 9500, GE Healthcare). The same procedure was carried out for the 5S rRNA ^{32}P 5'- end labelled oligonucleotide (I26, see Table 5), but with 2.5 h hybridization time and 45 minutes exposure time. All signals were quantified with ImageQuant (Molecular Dynamics) and the gene-specific signals were normalized to the 5S rRNA signals.

3.3.1 Oligonucleotide labeling

10 pmol of the respective oligonucleotide (V28, PaeII; W28, PaeIII or T28, RsmY, Table 5) were labeled with [γ-^{32}P] ATP, using the T4 polynucleotide kinase (Thermo Scientific). The probe was incubated at 37°C for 1 h, purified with the QIAquick Nucleotide Removal Kit (Qiagen) and stored at - 20°C.

3.3.2 Determination of the intracellular PaeII concentration

PAO1 and PAO1Δ*paeII* (Table 3) were grown to an OD_{600} of 1 in LB medium or BM2 medium (2.5.1) supplemented with 20 mM succinate. Samples of PAO1 grown in BM2 medium supplemented with 20 mM succinate were serially diluted and plated on LB plates

to determine the colony forming units (CFU). To determine the PaeII concentration, total RNA was isolated as described in 3.1. The samples (4 µg total RNA) were separated on a denaturating 8% polyacrylamide 8 M urea gel (2.6.2) together with 0.3, 0.6 and 1.2 ng of purified *in vitro* transcribed full length PaeII RNA (108 nt; the oligonucleotides L57 and L29 were used for amplification; Table 5). A Northern-blot analysis was performed as described in 3.3. For the detection of PaeII, the ^{32}P 5'- end labelled oligonucleotide V28 (Table 5) was used, as well as the 5S rRNA oligonucleotide I26 (Table 5) (internal control). The PaeII concentrations were determined with the aid of defined amounts of PaeII RNA loaded on the gel. The PaeII concentrations were then normalized to the CFU/ml and multiplied with the Avogadro constant resulting in the amount of PaeII molecules/cell.

3.4 Construction of plasmids

The following plasmids were constructed using standard molecular cloning techniques (Sambrook and Russell, 2001). All DNA inserts of the recombinant plasmids were verified by DNA sequencing (Mycrosynth).

3.4.1 pME4510*tacpaeIII*

First, a 98 bp DNA fragment encoding the PaeIII sRNA (genomic location: 3958053 – 3958150) was amplified from chromosomal PAO1 DNA with the oligonucleotides A121 and C121 (Table 5). The 120 nt PCR product was purified with the Quiagen PCR purification kit. The vector pME4510lacIQpTAClacO (Table 4) was isolated from *E. coli* TOP10 (Table 2), using the GeneJet Plasmid Miniprep Kit from Thermo Scientific. Subsequently, the *paeIII* fragment was digested with *Pst*I and *Sma*I and ligated into the corresponding sites of pME4510lacIQpTAClacO, resulting in pME4510*tacpaeIII.* The plasmid pME4510 *tacpaeIII* comprises the *paeIII* gene under transcriptional control of the *tac* promoter. The overproduction of the sRNA PaeIII was verified by Northern-blot analysis (Figure 27).

3.4.2 pME6016*dksA2-lacZ*

To construct a transcriptional *dksA2-lacZ* fusion a 705 bp fragment (genomic location: 6230576 – 6229872) containing the *dksA2* promoter region was amplified with primers D124 and E124 (Table 5), followed by purification with the PCR purification kit from Qiagen. The vector pME6016 (Table 4) was isolated from *E. coli* DH5α (Table 2) with the GeneJet Plasmid Miniprep Kit (Thermo Scientific) and purified by phenol-chloroform extraction after restriction with *EcoR*I and *Pst*I. The PCR product was cleaved with *EcoR*I and *Pst*I and ligated into the corresponding sites of pME6016, resulting in pME6016 *dksA2-lacZ.*

3.4.3 pME6016*rpoN-lacZ*

To construct a transcriptional *rpoN-lacZ* fusion a 378 bp fragment (genomic location: 4992453 – 4992830) was amplified by PCR with primers F124 and G124 (Table 5). The vector pME6016 (Table 4) was isolated from *E. coli* DH5α (Table 2) with the GeneJet Plasmid Miniprep Kit (Thermo Scientific) and purified by phenol-chloroform extraction after restriction with *EcoR*I and *Pst*I. The PCR product was processed with the Qiagen PCR purification kit and cleaved with *EcoR*I and *Pst*I prior to ligation into the corresponding sites of pME6016, resulting in pME6016*rpoN-lacZ.*

3.5 Transformation of *E. coli*

3.5.1 $CaCl_2$ competent cells

0.5 ml overnight culture of the *E. coli* strain DH5α (Table 2) were inoculated in 100 ml LB medium and grown to an optical density (OD_{600}) between 0.4 and 0.7 at 37°C. The cells were kept on ice for 10 minutes, before harvesting by centrifugation at 4,000 rpm for 5 minutes at 4°C. They were resuspended in 25 ml cold 0.1 M $CaCl_2$ solution and incubated on ice for 1 h, followed by centrifugation at 4,000 rpm for 5 minutes at 4°C. Then, the cells were washed with 10 ml $CaCl_2$ and centrifuged at 4,000 rpm for 5 minutes at 4°C. The pellet was resuspended in 1 ml $CaCl_2$ and 1 ml cold 50% glycerol. 100 µl aliquots were withdrawn and stored at - 80°C.

3.5.2 Transformation

Chemically competent DH5α cells (Table 2) were thawed on ice, 10 µl ligation reaction or 100 ng plasmid were added and the cells were incubated for 30 minutes on ice. The mix was heated for 2 minutes at 42°C, prior to addition of 1 ml LB (2.5.1) and regeneration at 37°C for 1–3 h with shaking and were plated on agar plates containing the appropriate antibiotics.

3.6 Transformation of *P. aeruginosa*

3.6.1 Electrocompetent cells

10 ml PAO1 (Table 3) overnight culture grown at 43°C with shaking were centrifuged at 6,000 rpm for 5 minutes at 4°C, and the pellet was resuspended in 2 ml glycerol/MOPS solution (2.6.3). A centrifugation step (8,000 rpm, 2 minutes, 4°C), followed by resuspension in 2 ml glycerol/MOPS solution, was performed. The cells were centrifuged again at

8,000 rpm for 2 minutes at 4°C, resuspended in 1 ml glycerol/MOPS solution and 100 µl aliquots were stored at - 80°C.

3.6.2 Transformation

100 ng plasmid were added on ice to 100 µl electrocompetent PAO1 (Table 3) cells and transferred to a sterile electroporation cuvette (VWR). Electroporation was performed at 200 Ω; 25 µF; 2.5 V with a BioRad Gene Pulser. The cells were regenerated in 1 ml LB (2.5.1) for 1–3 h at 37°C with shaking and were plated on agar plates containing the appropriate antibiotics.

3.7 Growth and colony forming units (CFU)

3.7.1 Zinc limitation assay

3.7.1.1 96-well plates growth curve

Overnight cultures of PAO1 and PAO1Δ*paeII* (Table 3) were inoculated in 14 ml polypropylene round bottom tubes (Eppendorf) and grown for 16 h with shaking at 37°C in BM2 minimal medium supplemented with 20 mM succinate (2.5.1). BM2 medium was prepared in plastic tubes, as glass flasks are a source of zinc contamination (D'Orazio *et al.*, 2015). TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylenediamine) was added to the minimal medium to ensure zinc limitation. TPEN is a cell permeable, high affinity heavy metal chelator (Zn^{2+} > Fe^{2+} > Mn^{2+}), which does not influence Mg^{2+} and Ca^{2+} concentrations (Arslan *et al.*, 1985). For growth monitoring, the overnight cultures were diluted to an initial OD_{600} of 0.1 and were grown in flat bottom 96-well plates (Sterilin) with shaking at 37°C. OD_{600} measurements were performed every hour with an iMark Microplate Absorbance Reader (BioRad).

3.7.1.2 Colony forming units

Overnight cultures of PAO1 and PAO1Δ*paeII* (Table 3) were inoculated in 14 ml polypropylene round bottom tubes (Eppendorf) and grown for 16 h with shaking at 37°C in BM2 minimal medium (2.5.1) supplemented with 20 mM succinate or 20 mM mannitol. The optical density was measured at 600 nm and the cultures were diluted to an initial OD_{600} of 1 before preparing serial dilutions (10^{-1} to 10^{-6}). 10 µl of the selected dilutions were spotted with a Gilson pipette on BM2 minimal medium plates (10^{-1}, 10^{-4}, 10^{-5} and 10^{-6}), either supplemented with 20 mM succinate or 20 mM mannitol. TPEN was added at a final concentration of 50 µM to the zinc limitation plates and additionally $ZnSO_4$, at a final

concentration of 50 µM, to complement the effect when necessary. The plates were incubated at 37°C, CFU were counted and pictures were taken on four successive days. Each assay contained three biological and four technical replicates of PAO1 and PAO1Δ*paeII*.

3.7.2 Nitrogen limitation assay

PAO1 and PAO1Δ*paeII* (Table 3) were grown in M9 minimal medium (2.5.1) with different concentrations of NH_4Cl. Overnight cultures were inoculated in M9 minimal medium supplemented with 19 mM NH_4Cl and washed twice in 0.9% NaCl (4°C, 4,000 rpm, 6 min), before shift to nitrogen starvation conditions. Final concentrations of 3 mM, 1.5 mM, 0.75 mM and 0.375 mM NH_4Cl were used to determine the nitrogen concentration at which the cells are growth impaired. To monitor a potential growth defect of PAO1Δ*paeII*, concentrations of 2.5 mM and 2 mM NH_4Cl were chosen. Three biological replicates were assayed for each strain.

3.7.3 Growth at 43°C

PAO1 and PAO1Δ*paeIII* (Table 3) were grown in LB medium at 37°C to an OD_{600} of 1. Then, the cultures were shifted to 43°C and the controls remained at 37°C. The samples were collected after 15, 45 and 75 minutes to prepare serial dilutions for CFU determination. 10 µl of these dilutions, in a range from 10^{-1} to 10^{-9}, were spotted on LB plates, which were incubated overnight at 37°C. Two biological and five technical replicates were used for each strain. Growth was monitored by measuring the optical density at 600 nm.

3.8 Western-blot analysis

3.8.1 Fluorescent Western-blotting

The cells were grown in LB (2.5.1). Samples were taken at the respective OD_{600} and centrifuged at 13,000 rpm for 2 minutes. The pellets were resuspended 1:1 in ddH_2O and 2x Laemmli buffer (2.6.4), before heating for 5 minutes at 100°C. The proteins were separated on a 12% SDS-polyacrylamide gel (2.6.4), and transferred to a nitrocellulose membrane (Amersham Protran, GE Healthcare Life Sciences) by electroblotting. The membrane was blocked overnight at 4°C in TBS with 5% milk powder (2.6.4). After blocking the membrane overnight, the blots were probed with anti-RpoS antibodies for 1 h at room temperature, respectively (dilution 1: 10,000 in TBSM; Table 6). The ribosomal proteins S2 (dilution 1: 10,000 in TBSM; Table 6) and S4 (dilution 1: 1,000 in TBSM; Table 6) were used as internal controls. The blots were probed for 1 h at room temperature with anti-S2 antibodies and overnight at 4°C with anti-S4 antibodies. The membrane was washed in

TBST (2.6.4) and the antibody-antigen complex was visualized with the Rockland IRDye 800 conjugated anti-goat or anti-rabbit IgG (dilution 1: 15,000 in TBSM; Table 6). The blots were probed with Rockland IRDye 800 conjugated anti-goat or anti-rabbit IgG for 30 minutes at room temperature. The membrane was washed again in TBST prior to signal detection with the Odyssey Infrared Imaging System. Afterwards, the signals were removed by treating the membrane twice with stripping buffer (2.6.4) for 20 minutes. This process allows the detection of additional signals from the same membrane. Quantification was performed with the program ImageQuant (Molecular Dynamics).

3.9 β-Galactosidase assay

3.9.1 Growth conditions

P. aeruginosa shows severe growth defects in BM2 minimal medium supplemented with 20 mM mannitol. Moreover, a PAO1 *rpoN* mutant strain shows a general growth defect in minimal medium (Valentini *et al.*, 2011). It was not possible to obtain overnight pre-cultures of PAO1 in BM2 minimal medium supplemented with 20 mM mannitol or of PAO1Δ*rpoN* in BM2 minimal medium supplemented with 20 mM succinate or 20 mM mannitol. Therefore, the cells were grown in LB medium before shift to minimal medium. Two different methods were applied: first, the pre-cultures were directly shifted to minimal medium after overnight growth (3.9.1.1), or second, the pre-cultures were used for inoculation in LB medium and the cells were harvested in exponential phase (OD_{600} of 1), before shift to minimal medium (3.9.1.2).

3.9.1.1 Shift in stationary phase

Pre-cultures were grown overnight in LB medium before the cells were washed twice in 0.9% NaCl (4°C, 4,000 rpm, 6 min) to remove LB medium. Subsequently, the cells were shifted to BM2 minimal medium supplemented with 20 mM succinate or 20 mM mannitol. Samples for β-galactosidase measurements were taken during exponential phase (OD_{600} of 1) and stationary phase (OD_{600} of 2).

3.9.1.2 Shift in exponential phase

Pre-cultures were grown overnight in LB medium and were used for inoculation in LB medium. The cells were harvested at an OD_{600} of 1 and were washed twice with 0.9% NaCl (4°C, 4,000 rpm, 6 min) to remove LB medium before shift to BM2 medium supple-

mented with 20 mM succinate or 20 mM mannitol. Samples for β-galactosidase measurements were taken during exponential phase (OD_{600} of 1) and stationary phase (OD_{600} of 2).

3.9.2 Experimental procedure

The β-galactosidase measurements were carried out as described by Miller (1972). 1 ml samples were withdrawn at different optical densities (OD_{600}) during growth in given media (see 3.9.1). After centrifugation at 13,000 rpm for 2 minutes, the pellets were resuspended in 0.9% NaCl to measure the OD_{600}. The cells were vortexed for 20 seconds after addition of 0.05 volume toluene and incubated at 37°C for 10 minutes before they were lysed overnight at 4°C. The lysates were diluted (1:10) with Z-buffer (2.6.5) and incubated at 37°C for at least 5 minutes. The reaction was started by adding 200 µl ONPG (4 mg/ml) (o-Nitrophenyl-β-D-galactopyranosid) and stopped with 500 µl 1 M Na_2CO_3. The supernatant was measured at OD_{420} with a spectrophotometer (Hitachi U-1100). The Miller Units (MU) were calculated using the following formula: MU= (1000 x OD_{420}) / t (minutes) x V x OD_{600}. V= 0.1, the lysates were diluted 1:10 with Z-buffer. All experiments were performed in duplicate.

3.10 Antibiotic susceptibility tests

3.10.1 Disc diffusion assays

3.10.1.1 Antimicrobial Susceptibility Test Discs

PAO1 and PAO1Δ*paeIII* (Table 3) were grown in LB medium to an OD_{600} of 2. 200 µl bacterial culture were plated on dried LB agar plates (2.5.1). Then, the Antimicrobial Susceptibility Test Discs (OXOID), containing 30 µg/ml tetracycline, were applied with sterile forceps. Before evaluating the inhibition zone, the plates were incubated at 37°C for 12 hours.

3.10.1.2 Minimum Inhibitory Concentration Evaluator Strips

The cells were grown in LB medium to an OD_{600} of 2. 200 µl of each culture were plated on dried LB agar plates (2.5.1). The Minimum Inhibitory Concentration Evaluator Strips (OXOID), covering a tetracycline range from 0.015 µg/ml - 256 µg/ml, were placed on the plates with sterile forceps. The minimal inhibitory concentration (MIC) was evaluated after an incubation time of 12 hours at 37°C.

3.11 Microtiter plate biofilm assay

Biofilm formation of PAO1 and PAO1Δ*paeIII* (Table 3) was assessed as described (Merritt *et al.*, 2005). Both strains were grown overnight in LB (2.5.1) at 37°C. The cultures were diluted to an initial OD_{600} of 0.05. The bacteria were incubated in LB medium for 24 and 48 hours, under aerobic and anaerobic conditions, using flat bottom 96-well plates (Sterilin). To create an anaerobic environment, the cells were grown in an anaerobic jar with gas packs and 100 mM KNO_3 was added to the medium. The biofilm was stained with 0.1% crystal violet solution and solubilized with 96% ethanol. The crystal violet/ethanol solution was transferred to an optically clear flat-bottom 96-well plate and was quantified with an iMark Microplate Absorbance reader (BioRad) at a wavelength of 595 nm.

4 Results

4.1 PaeII

4.1.1 PaeII abundance in minimal medium

The intracellular abundance of PaeII (3.3.2) was determined in wild-type cells grown in rich and minimal medium. PAO1 was grown in LB medium and BM2-succinate to an OD_{600} of 1. Then, samples were collected for RNA isolation and CFU determination. A Northern-blot was performed to assess the amount of PaeII per cell. No PaeII signal could be detected in LB medium, whereas high levels were observed in samples grown in BM2-succinate medium (Figure 13). In BM2 medium supplemented with 20 mM succinate, the intracellular concentration of PaeII was determined with 1400 molecules per cell.

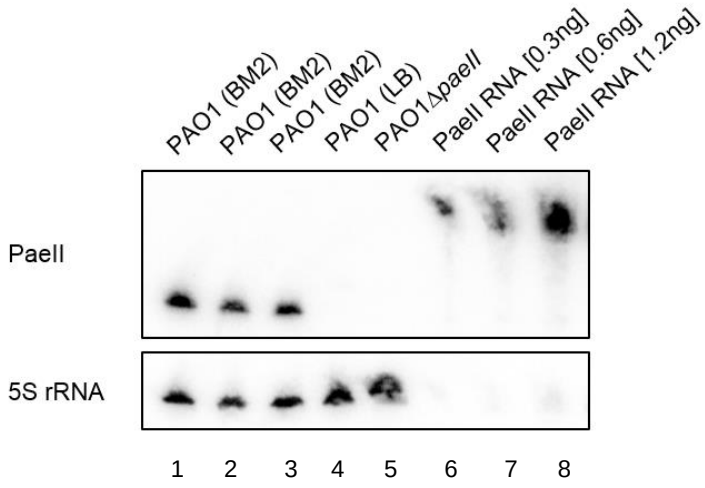


Figure 13. Determination of the cellular PaeII concentration by Northern-blot analysis.
PAO1 was grown in BM2-succinate medium and LB medium to an OD_{600} of 1 (≙ 7.0 ± 1.4 10^7 CFU). PAO1Δ*paeII* was included as a negative control. Three samples were taken from three individual cultures to determine the PaeII concentration. The RNA samples (4 µg total RNA) were separated on a denaturating 8% polyacrylamide 8 M urea gel (2.6.2) together with 0.3, 0.6 and 1.2 ng *in vitro* transcribed full length PaeII RNA (108 nt*;* lanes 6-8). Only the 76 nt processed form (Sorger-Domenigg, 2010) of PaeII is visible (lanes 1-3). Total RNA was prepared from three biological replicates of PAO1 (lanes 1-3) and of PAO1Δ*paeII* (lane 5) grown in BM2 medium supplemented with 20 mM succinate, as well as of PAO1 cells grown in LB medium (lane 4). The PaeII concentration was determined as described in 3.3.2.

4.1.2 Elucidating the enhancer-binding protein that initiates *paeII* transcription

The steady state levels of PaeII were ~ 80% reduced when mutations were introduced in the putative σ^{54}(RpoN) promoter region (Sorger-Domenigg, 2010). This result mirrored the decreased steady state levels observed in a PAO1 *rpoN* mutant strain (Sorger-Domenigg, 2010). Thus, *paeII* transcription appears to be to a great extent σ^{54}-dependent. Unlike the housekeeping RNAP sigma factor RpoD (σ^{70}), the alternative sigma factor RpoN requires EBPs to exert its function (Bush and Dixon, 2012). The EBP that is required for RpoN-dependent *paeII* transcription is hitherto unknown. The DNA-binding protein NtrC is a known EBP (Porter *et al.*, 1995) and the response regulator of the NtrB/C two-component system (Contreras and Drummond, 1988; Stock *et al.*, 1989). Upstream of the *paeII* promoter sequence are two potential NtrC-binding sites (Figure 14).

CbrB is the response regulator of the CbrA/B two-component system and another EBP for RpoN-dependent transcription (Abdou *et al.*, 2011). The CbrA/B system regulates the carbon-nitrogen balance in the cell (Nishijyo *et al.*, 2001). However, CbrB is less likely to control *paeII* transcription, as no potential CbrB recognition motif was identified upstream of the *paeII* gene. A Northern-blot analysis with total RNA extracted from a PAO1Δ*ntrC* and a PAO1Δ*cbrB* strain was performed to test whether either EBP can affect *paeII* transcription. As expected, the PaeII levels were significantly reduced in the PAO1Δ*rpoN* strain (Figure 15A, lane 4 and Figure 15B, lane 4), emphasizing that the major part of *paeII* transcription relies on a σ^{54} RNAP holoenzyme. The amount of PaeII was similar in

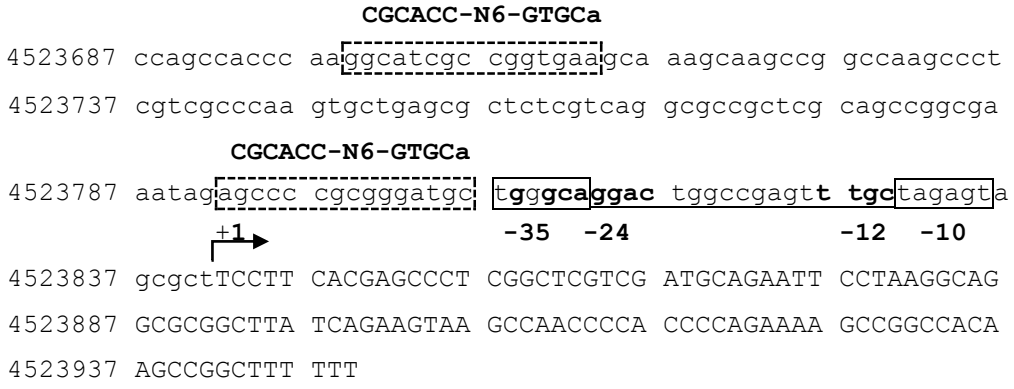


Figure 14. Localisation of putative NtrC-binding sites upstream of the *paeII* gene.

The *paeII* sequence (uppercase letters), including 155 bp upstream (lowercase letters) of the transcription start site (arrow), is depicted. The σ^{54} recognition site with -24 and -12 regions is underlined and the σ^{70} promoter sequence with -35 and -10 regions is boxed. The two putative NtrC-binding sites are marked with dotted boxes. The NtrC consensus sequence CGCACC-N6-GTGCa (Hervas *et al.*, 2008) is shown above. The numbers on the left represent the corresponding genomic coordinates.

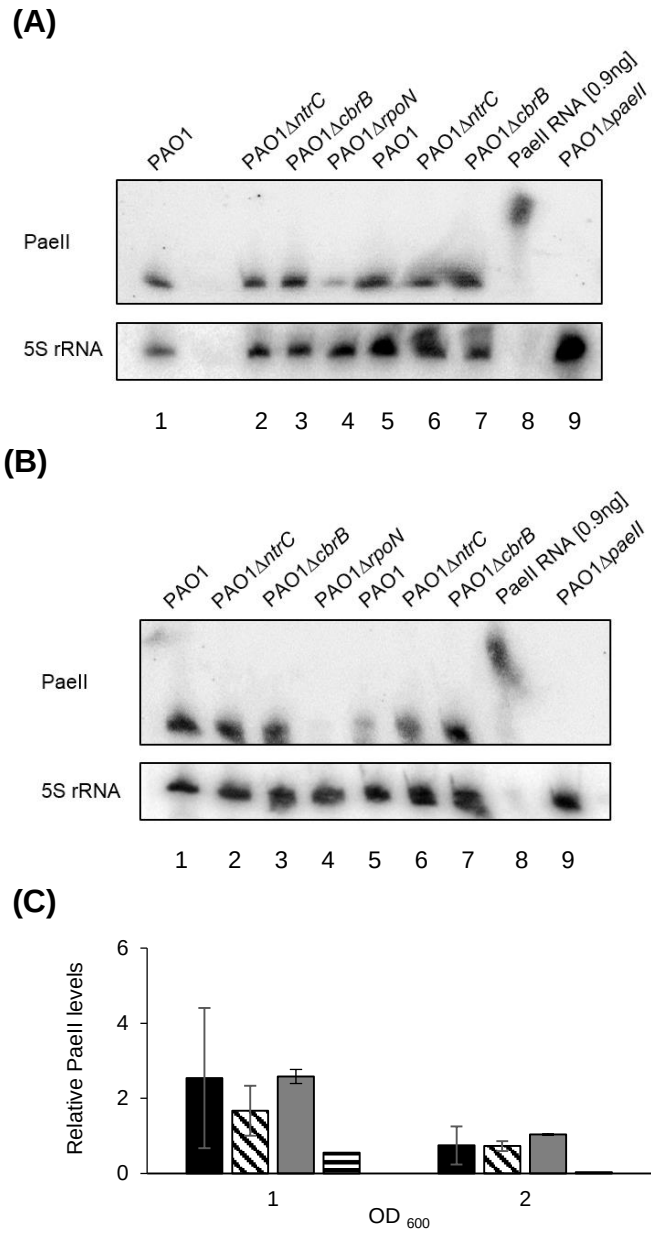


Figure 15. NtrC is not required for *paeII* transcription.

(A and B) PAO1, PAO1Δ*ntrC,* PAO1Δ*cbrB,* PAO1Δ*rpoN* and PAO1Δ*paeII* were grown in BM2 minimal medium supplemented with 20 mM succinate and 0.2% L-glutamine. The samples for RNA isolation were withdrawn at an OD_{600} of 1 **(A)** and 2 **(B)**. The PaeII levels were determined by Northern-blot analysis using the ^{32}P 5'- end labelled oligonucleotides V28 (PaeII) and I26 (5S rRNA; loading control). *In vitro* transcribed full length PaeII RNA (0.9 ng; lane 8) and PAO1Δ*paeII* were used as controls. Total RNA was prepared from two biological replicates of PAO1 (lanes 1 and 5), PAO1Δ*ntrC* (lanes 2 and 6) and PAO1Δ*cbrB* (lanes 3 and 7). Total RNA prepared from the strains PAO1Δ*rpoN* (lane 4) and PAO1Δ*paeII* (lane 9) was used as a control. **(C)** Relative PaeII levels of the strains PAO1 (black bars), PAO1Δ*ntrC* (tilted striped bars), PAO1Δ*cbrB* (grey bars) and PAO1Δ*rpoN* (horizontally striped bars) at an OD_{600} of 1 and 2 normalized to the 5S rRNA signal. The ImageQuant software (Molecular Dynamics) (3.3) was used for quantification. The error bars represent standard deviations for two biological replicates.

the strains PAO1, PAO1Δ*ntrC* and PAO1Δ*cbrB*, which suggests that neither NtrC (Figure 15A and B, lanes 2 and 6) nor CbrB (Figure 15A and B, lanes 3 and 7) is the enhancer-binding protein that accounts for activation of *paeII* transcription by RpoN.

4.1.3 PaeII is not required for growth during nitrogen limitation

As *paeII* transcription is to a great extent σ^{54}-dependent (Figure 15A and B, lane 4) (Sorger-Domenigg, 2010), it was determined whether PaeII might be required for growth during nitrogen depletion. PAO1 and PAO1Δ*paeII* were grown overnight in M9 minimal medium supplemented with 19 mM NH_4Cl. The pre-cultures were washed with 0.9% NaCl to remove traces of nitrogen and the cells were resuspended in M9 medium supplemented with 2 mM or 2.5 mM NH_4Cl. It was examined whether PAO1Δ*paeII* displays a delayed growth phenotype during NH_4Cl limitation. As shown in Figure 16A and B, PAO1Δ*paeII* was not impaired in growth during nitrogen limitation when compared with the wild-type strain.

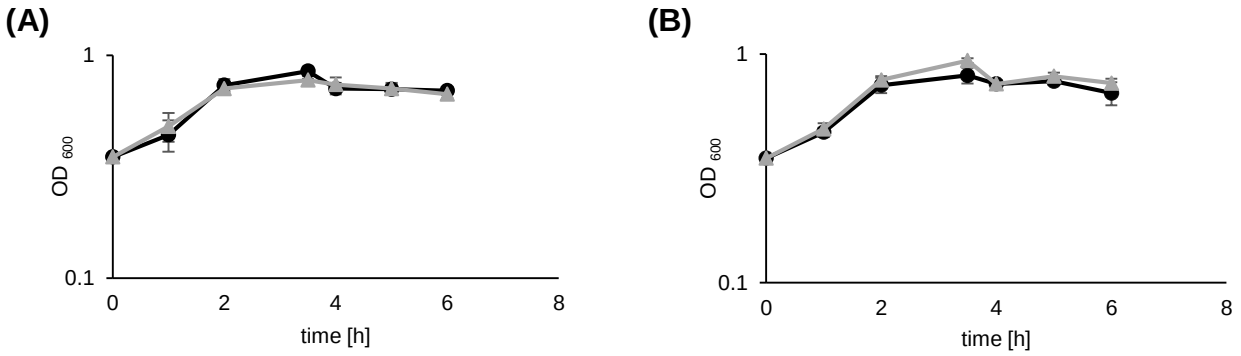


Figure 16. PAO1Δ*paeII* is not impaired in growth during nitrogen limitation.

(A and B) Overnight cultures of PAO1 and PAO1Δ*paeII* were grown in M9 minimal medium supplemented with 19 mM NH_4Cl. Excess nitrogen was removed by washing the cells twice with 0.9% NaCl (3.7.2.). Subsequently, PAO1 (black dots) and PAO1Δ*paeII* (grey triangles) were inoculated in M9 minimal medium either supplemented with 2 mM NH_4Cl **(A)** or 2.5 mM NH_4Cl **(B)**. The error bars represent standard deviations for two biological replicates.

4.1.4 A role of PaeII in zinc homeostasis?

Rindler (2014) performed a comparative transcriptome analysis with strains PAO1Δ*paeII* (pJT19) and PAO1Δ*paeII*(pJT*paeII*) grown in BM2-succinate medium. These RNA-seq data revealed that *dksA2* and 33 other genes involved in zinc homeostasis (Pederick *et al.*, 2015) were up-regulated upon *paeII* over-expression. Thus, it was considered that PaeII might indirectly influence Zur-regulated transcripts *via* a transcriptional regulator

like Zur or RpoN. Another possibility was that PaeII might affect the intracellular Zn^{2+} levels.

4.1.4.1 PaeII does not impact on growth during zinc depletion

First, the growth phenotypes of PAO1 and PAO1Δ*paeII* were compared during zinc depletion. The cells were grown for eight hours in 96-well plates (3.7.1.1) containing BM2 minimal medium supplemented with 20 mM succinate. One half of the cultures was treated with the zinc chelating agent TPEN (3.7.1). A pronounced reduction in the growth rates was observed for both strains in zinc-depleted medium (Figure 17B) relative to BM2 medium (Figure 17A). Nevertheless, zinc limitation had no specific impact on PAO1Δ*paeII* when compared with the wild-type strain (Figure 17B). To further test a potential role of PaeII on cell viability during zinc limitation, the CFUs were determined as described in 3.7.1.2. PAO1 and PAO1Δ*paeII* were grown in 14 ml polypropylene round bottom tubes in BM2 minimal medium supplemented with 20 mM succinate (Rindler, 2014) (Figure 18A and C) or 20 mM mannitol (Sorger-Domenigg, 2010) (Figure 18B and C). TPEN was added to the medium at a final concentration of 50 µM to ensure zinc depletion. For complementation, the same amount of $ZnSO_4$ was used. Both the wild-type and the PAO1Δ*paeII* strain reached the same CFUs independent of the used carbon source (Figure 18A and B), suggesting that PaeII is not essential for growth during zinc limitation.

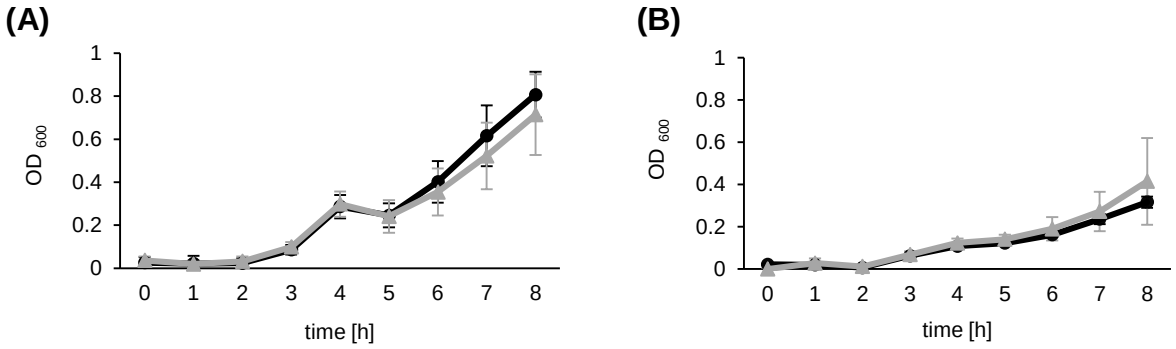


Figure 17. PAO1Δ*paeII* is not impaired in growth during zinc depletion.
(A) PAO1 (black dots) and PAO1Δ*paeII* (grey triangles) were grown in 96-well plates containing BM2 medium (20 mM succinate) and growth was monitored with an iMark Microplate Absorbance Reader (BioRad) for eight hours. (**B)** PAO1 (black squares) and PAO1Δ*paeII* (grey triangles) were cultured in BM2 medium supplemented with 20 mM succinate and 50 µM of the heavy metal chelator TPEN. The error bars represent standard deviations for two biological replicates.

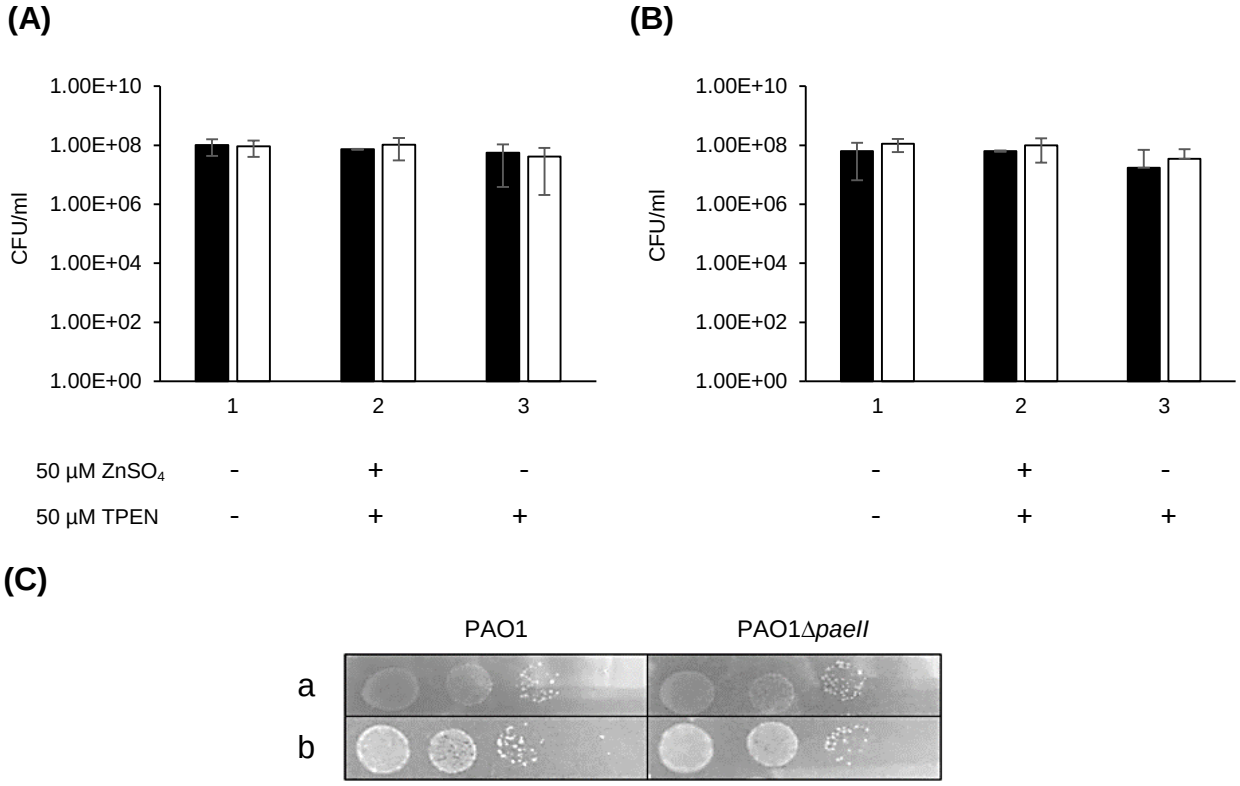


Figure 18. PaeII has no impact on cell viability during zinc depletion.

(A and B) PAO1 (black bars) and PAO1Δ*paeII* (white bars) were grown overnight in BM2 minimal medium supplemented with 20 mM succinate **(A)** or 20 mM mannitol **(B)**. The cells were diluted to an initial OD_{600} of 1 followed by serial dilutions from 10^{-1} to 10^{-6}. **(C) a**, PAO1 and PAOΔ*paeII* were grown in BM2 medium supplemented with 20 mM succinate and 50 µM TPEN; **b**, PAO1 and PAOΔ*paeII* were grown in BM2 medium supplemented with 20 mM mannitol and 50 µM TPEN. Pictures were taken from serial dilutions 10^{-1}, 10^{-4}, 10^{-5} and 10^{-6} (from left to right) after 4 days of growth at 37°C. The error bars represent standard deviations for two independent experiments.

4.1.4.2 *DksA2* levels are indistinguishable in the presence and absence of PaeII

RNA sequencing data (Rindler, 2014) revealed that many Zur-regulated genes were up-regulated upon *paeII* over-expression. Among them was the transcriptional regulator DksA2. *DksA2* transcription was 12.3-fold up-regulated in PAO1Δ*paeII*(pJT*paeII*) in BM2 medium containing 20 mM succinate. To verify the RNA-seq data, a Real-time quantitative PCR was performed to examine *dksA2* transcript levels under the same conditions as were used for the RNA-seq experiment. The *dksA2* transcript levels were determined in PAO1, PAO1Δ*paeII*, PAO1Δ*paeII*(pJT19) and PAO1Δ*paeII*(pJT*paeII*), respectively. The *dksA2* transcript levels observed for PAO1Δ*paeII* were the same as those obtained for the wild-type strain. Likewise, PAO1Δ*paeII*(pJT*paeII*) showed no altered *dksA2* levels

when compared with the control strain PAO1Δ*paeII*(pJT19) (Figure 19). The 12-fold higher *dksA2* levels observed for PAO1Δ*paeII*(pJT*paeII*) in the transcriptome data (Rindler, 2014) could thus not be verified.

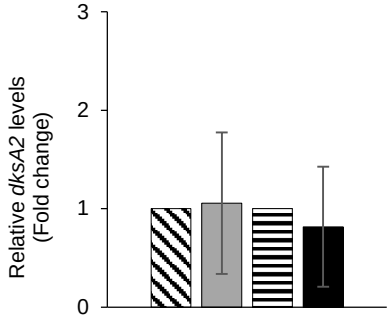


Figure 19. *DksA2* transcript levels are not enhanced upon *paeIII* over-expression.
PAO1 (tilted striped bar) and PAO1Δ*paeII* (grey bar) were grown in BM2 medium supplemented with 20 mM succinate and 0.1% Triton X-100 to an OD_{600} of 1. PAO1Δ*paeII*(pJT19) (horizontally striped bar) and PAO1Δ*paeII*-(pJT*paeII*) (black bar) were grown in BM2 minimal medium supplemented with 20 mM succinate, 0.1% Triton X-100 and 400 µg/ml kanamycin to an OD_{600} of 1. The *dksA2* transcript levels were observed with Real-time quantitative PCR. The relative transcript levels were calculated with the comparative C_T method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) and the results are represented as fold change. The *dksA2* levels found in PAO1Δ*paeII* and PAO1Δ*paeII (*pJT*paeII*) were normalized to the respective control strains PAO1 and PAO1Δ*paeII* (pJT19) (levels were set to 1). The error bars represent standard deviations for three biological replicates.

4.1.4.3 PaeII does not regulate *dksA2* expression

To clarify the contradictory results of the RNA-seq analysis and the RT-qPCR, the *dksA2* expression was monitored in the presence and absence of PaeII. Therefore, the β-galactosidase values conferred by transcriptional and translational fusions of *dksA2* to the *lacZ* reporter gene were determined. The 5' UTR of the *dksA2* gene contains a potential RpoN-dependent promoter sequence (Figure 20). Therefore, an *rpoN* deletion strain was included to investigate a potential σ^{54}-dependence of *dksA2*. The strains PAO1, PAO1Δ*paeII* and PAO1Δ*rpoN* were transformed with plasmid pME6016*dksA2-lacZ* harboring a transcriptional *dksA2-lacZ* fusion and with plasmid pME6015*dksA2::lacZ* harboring a translational *dksA2::lacZ* fusion, respectively. In addition, PAO1 was transformed with plasmid pME4510*paeII* harboring *paeII* under control of its authentic promoter or the empty plasmid pME4510 (control). Then, PAO1(pME4510*paeII*) and PAO1(pME4510) were transformed with pME6016*dksA2-lacZ* and pME6015 *dksA2::lacZ*, respectively. All strains were grown in BM2 minimal medium supplemented with 0.2% L-glutamine and 20 mM succinate or 20 mM mannitol. The medium was supplemented with 0.2% L-glutamine, as PAO1Δ*rpoN* is glutamine auxotroph (Totten *et al.*, 1990; Heurlier *et al.*, 2003).

Samples for β-galactosidase measurements were taken during exponential phase (OD_{600} of 1) and stationary phase (OD_{600} of 2). Due to the severe growth defect of PAO1Δ*rpoN* in minimal medium (Valentini *et al.*, 2011), the strains harboring pME6016*dksA2-lacZ* were grown as described in 3.9.1.1 and the strains harboring pME6015*dksA2::lacZ* were grown as described in 3.9.1.2. The β-galactosidase activity conferred by the transcriptional *dksA2-lacZ* fusion was comparable in PAO1, PAO1Δ*paeII*, PAO1(pME4510) and PAO1(pME4510*paeII*) (Figure 21A and B) grown in BM2 supplemented with succinate or mannitol. Surprisingly, in the PAO1Δ*rpoN* strain, the β-galactosidase activity was up to 5.3-fold higher than in the wild-type strain (Figure 21A, OD_{600} of 1), when the cells were grown in BM2-succinate. At least 8-fold higher values were observed in BM2-mannitol (Figure 21B, OD_{600} of 2). The *dksA2::lacZ* translation was comparable in all strains, independent of the used carbon source (Figure 21C and D). According to the data obtained with the *dksA2* reporter gene fusions, *dksA2* transcription is not RpoN-dependent (Figure 21A and B). Furthermore, these results are in line with the outcome of the RT-qPCR experiment. Hence, PaeII appears not to regulate *dksA2* expression (Figure 21C and D).

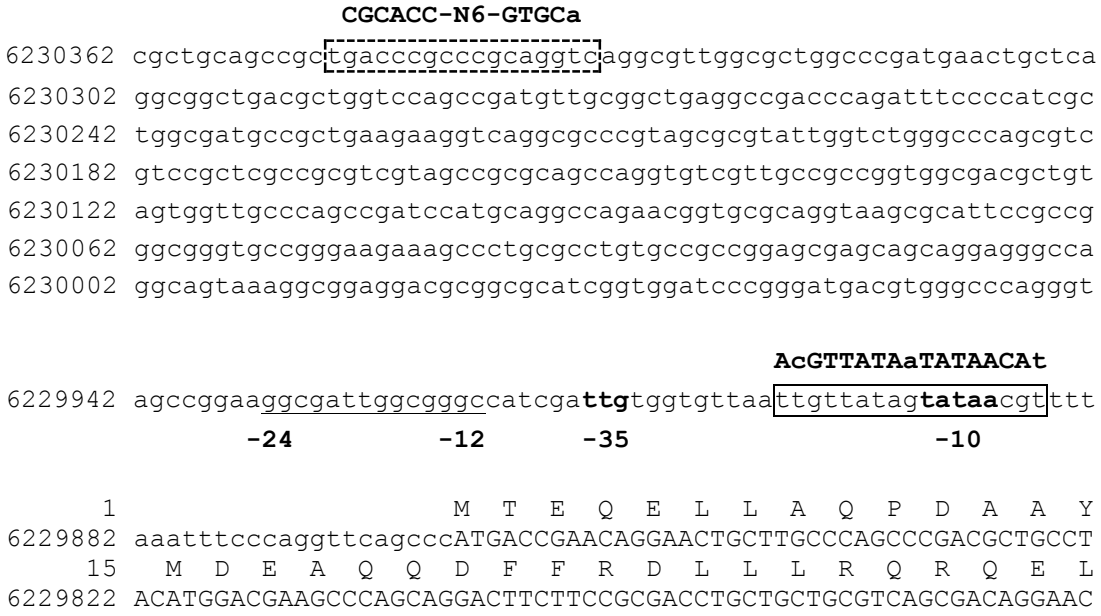


Figure 20. Sequence of the 5' UTR and the first 100 nucleotides of the *dksA2* gene.

Upstream sequences are depicted in lowercase letters, while the open reading frame is displayed in uppercase letters with the corresponding amino acid sequence above. The 5' UTR of *dksA2* contains a putative σ^{70}-promoter (in bold) and a potential σ^{54}-promoter (underlined) with a possible NtrC-binding site (dotted box), the NtrC consensus sequence (Hervas *et al.*, 2008) is shown above. The Zur-binding site proposed for PAO1 by Pederick *et al.* (2015) is boxed and overlaps the putative σ^{70}-promoter (consensus motif shown above). The numbers on the left represent the corresponding genomic coordinates.

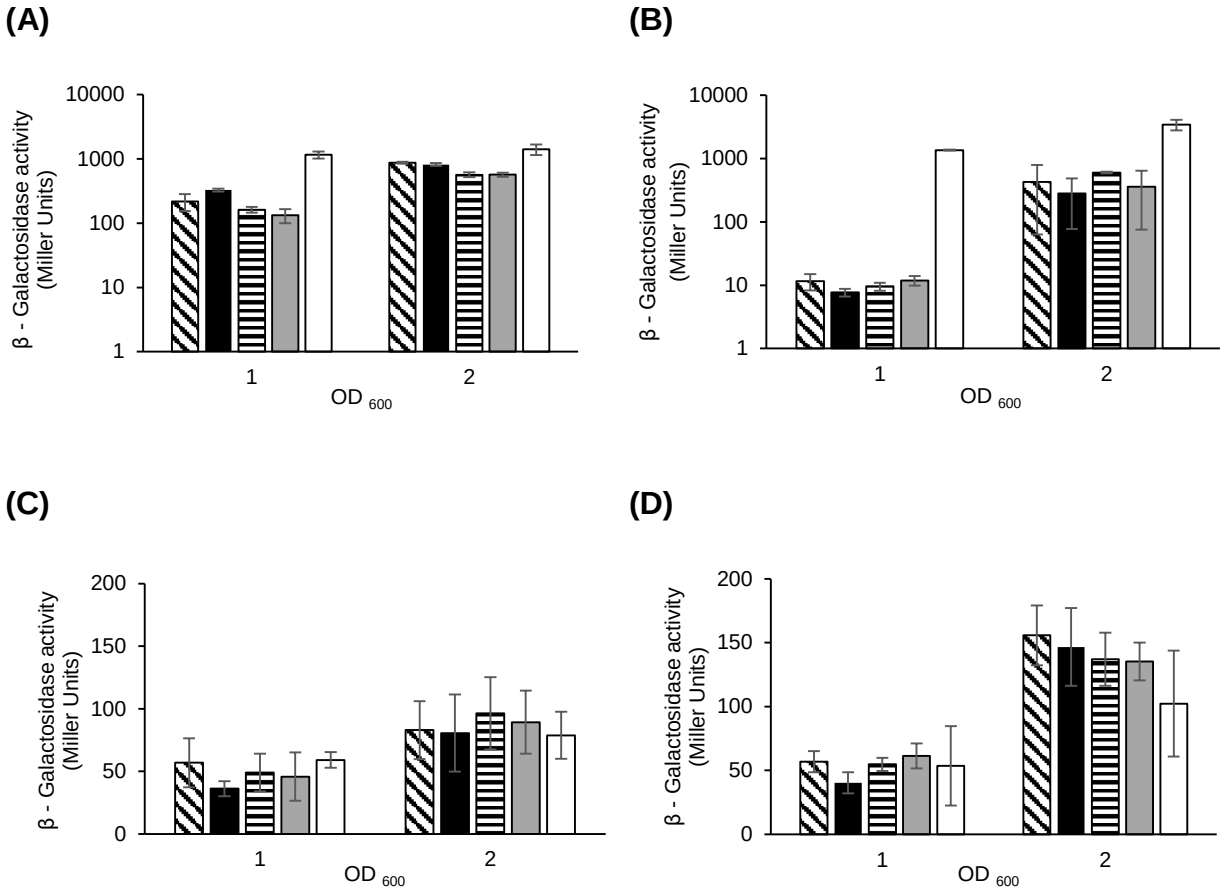


Figure 21. *DksA2* levels are not enhanced in the presence of PaeII.

(A and B) The strains PAO1(pME6016*dksA2-lacZ*) (tilted striped bars), PAO1Δ*paeII*(pME6016*dksA2-lacZ*) (black bars), PAO1(pME4510, pME6016*dksA2-lacZ*) (horizontally striped bars), PAO1(pME4510*paeII*, pME6016*dksA2-lacZ*) (grey bars) and PAO1Δ*rpoN*(pME6016*dksA2-lacZ*) (white bars) were grown in BM2 minimal medium supplemented with 20 mM succinate and 0.2% L-glutamine **(A)** or BM2 minimal medium supplemented with 20 mM mannitol and 0.2% L-glutamine **(B)** to an OD_{600} of 1 and 2. The bars represent the β-galactosidase values conferred by the plasmid pME6016*dksA2-lacZ* encoded transcriptional *dksA2-lacZ* fusion. The error bars represent standard deviations for two biological replicates. Due to the enormous differences in order of magnitude, the β-galactosidase values were depicted in a log scale. **(C and D)** The strains PAO1(pME6015*dksA2::lacZ*) (tilted striped bars), PAO1Δ*paeII*(pME6015*dksA2::lacZ*) (black bars), PAO1(pME4510, pME6015*dksA2::lacZ*) (horizontally striped bars), PAO1(pME4510*paeII*, pME6015*dksA2::lacZ*) (grey bars) and PAO1Δ*rpoN*(pME6015*dksA2::lacZ*) (white bars) were grown in BM2 minimal medium supplemented with 20 mM succinate and 0.2% L-glutamine **(C)** or BM2 minimal medium supplemented with 20 mM mannitol and 0.2% L-glutamine **(D)** to an OD_{600} of 1 and 2. The bars represent the β-galactosidase values conferred by the plasmid pME6015*dksA2::lacZ* encoded translational *dksA2::lacZ* fusion. The error bars represent standard deviations for two biological replicates.

4.1.5 PaeII does not regulate *rpoN* expression

Some sRNAs influence the translation of the sigma factor that is required for their expression. Consequently, they control their own expression *via* a feedback loop (Papenfort *et al.*, 2006; Udekwu and Wagner, 2007; Thompson *et al.*, 2007). It is possible that PaeII has a regulatory effect on *rpoN* translation, which might lead to enhanced *paeII* expression. Hence, the β-galactosidase values conferred by transcriptional and translational fusions of *rpoN* to the *lacZ* reporter gene were determined. The strains PAO1 and PAO1Δ*paeII* were transformed with plasmid pME6016*rpoN-lacZ* harboring a transcriptional *rpoN-lacZ* fusion and with plasmid pME9851 harboring a translational *rpoN::lacZ*

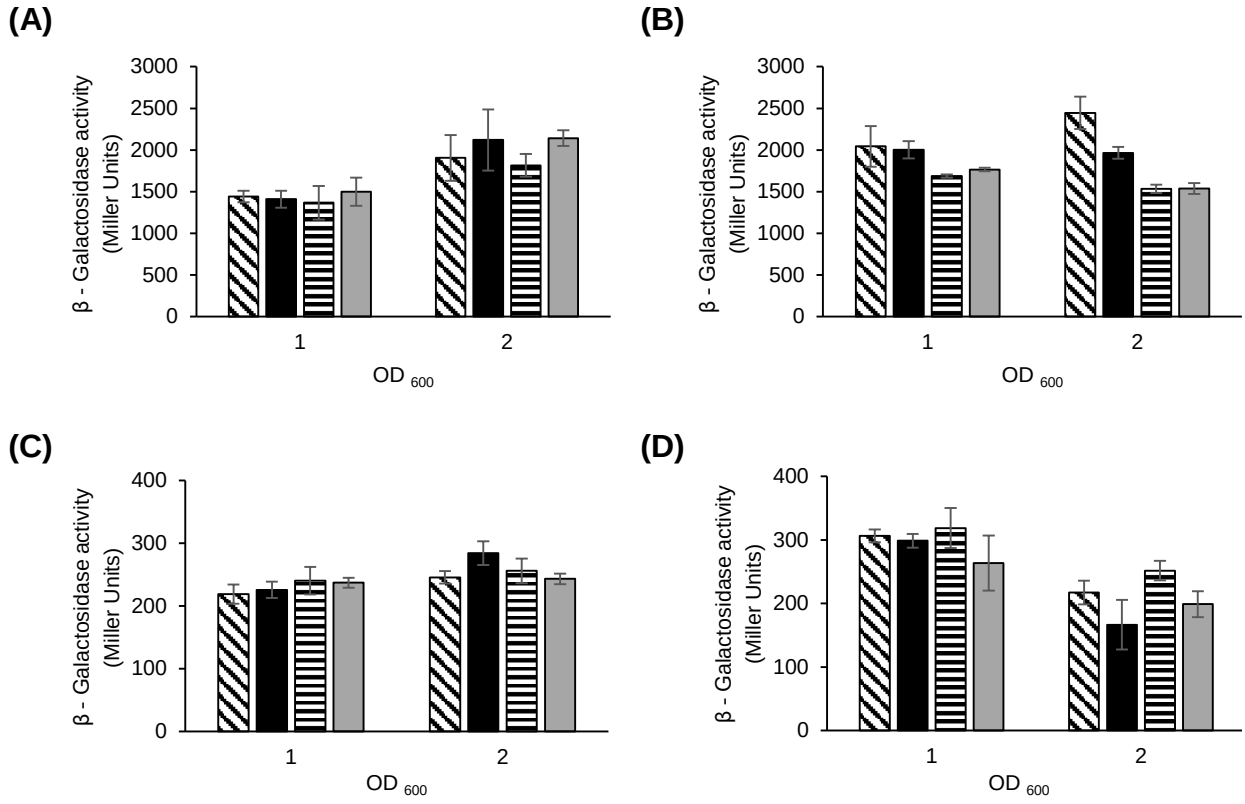


Figure 22. *RpoN* levels are not influenced by PaeII.

(A and B) PAO1(pME6016*rpoN-lacZ*) (tilted striped bars), PAOΔ*paeII*(pME6016*rpoN-lacZ*) (black bars), PAO1-(pME4510, pME6016*rpoN-lacZ*) (horizontally striped bars) and PAO1(pME4510*paeII*, pME6016*rpoN-lacZ*) (grey bars) were grown in BM2 minimal medium supplemented with 20 mM succinate **(A)** or with 20 mM mannitol **(B)** to an OD_{600} of 1 and 2. The bars represent the β-galactosidase values conferred by the plasmid pME6016*rpoN-lacZ* encoded transcriptional *rpoN-lacZ* fusion. The error bars represent standard deviations for two biological replicates. (**C and D)** PAO1(pME9851) (tilted striped bars), PAOΔ*paeII*(pME9851) (black bars), PAO1(pME4510, pME9851) (horizontally striped bars) and PAO1(pME4510*paeII*, pME9851) (grey bars) were grown in BM2 supplemented with 20 mM succinate **(C)** or 20 mM mannitol **(D)** to an OD_{600} of 1 and 2. The bars represent the β-galactosidase values conferred by the plasmid pME9851 encoded translational *rpoN::lacZ* fusion.The error bars represent standard deviations for two biological replicates.

fusion, respectively. In addition, PAO1 was transformed with plasmid pME4510*paeII* harboring *paeII* under its authentic promoter and the empty plasmid pME4510 was used as a control. Then, PAO1(pME4510*paeII*) and PAO1(pME4510) were transformed with pME6016*rpoN-lacZ* and pME9851, respectively. All strains were grown in BM2 minimal medium supplemented with 20 mM succinate or 20 mM mannitol. Samples for β-galactosidase measurements were taken during exponential phase (OD_{600} of 1) and stationary phase (OD_{600} of 2). As the cell growth is reclined in minimal medium with mannitol as the sole carbon source, the strains harboring pME6016*rpoN-lacZ* were grown as desribed in 3.9.1.2 and the strains harboring pME9851 were grown as described in 3.9.1.1. The *rpoN-lacZ* transcript levels were similar in all strains, independent of the carbon source and the growth phase (Figure 22A and B). These results mirrored those obtained by the translational *rpoN* reporter gene fusion (Figure 22C and D). The *rpoN::lacZ* translation was not altered in the presence or absence of PaeII independently of the tested carbon source. These results indicated that PaeII is not involved in *rpoN* expression. In addition, the transcript levels of the two RpoN-regulated sRNAs CrcZ and PhrS were determined. The plasmid encoded transcriptional *phrS-lacZ* and *crcZ-lacZ* fusions were in line with the results that were obtained by measuring RpoN-LacZ synthesis. The transcript levels of *phrS-lacZ* (Supplementary Figure S1A and B) and *crcZ-lacZ* (Supplementary Figure S2A and B) were not altered in the presence or absence of PaeII. The direct σ^{54}-dependence of *crcZ* transcription and the stimulatory effect of RpoN on *phrS* transcription does not correlate with the presence or absence of PaeII.

4.2 PaeIII

4.2.1 PaeIII abundance is increased upon *rsmA* deletion

The post-transcriptional regulator RsmA plays a pivotal role in the regulation of chronic and acute states of infection and is counteracted by the RsmY/Z RNAs (Pessi *et al.*, 2001). Sorger-Domenigg (2010) observed that the PaeIII levels in stationary phase were 2-fold up-regulated in the PAO1Δ*rsmA* strain when compared with the wild-type strain. RsmA prevents translation *via* binding to GGA-sequences in the 5' UTR of target mRNAs (Heeb *et al.*, 2004; Pessi *et al.*, 2001). There is no evidence that RsmA can act at the

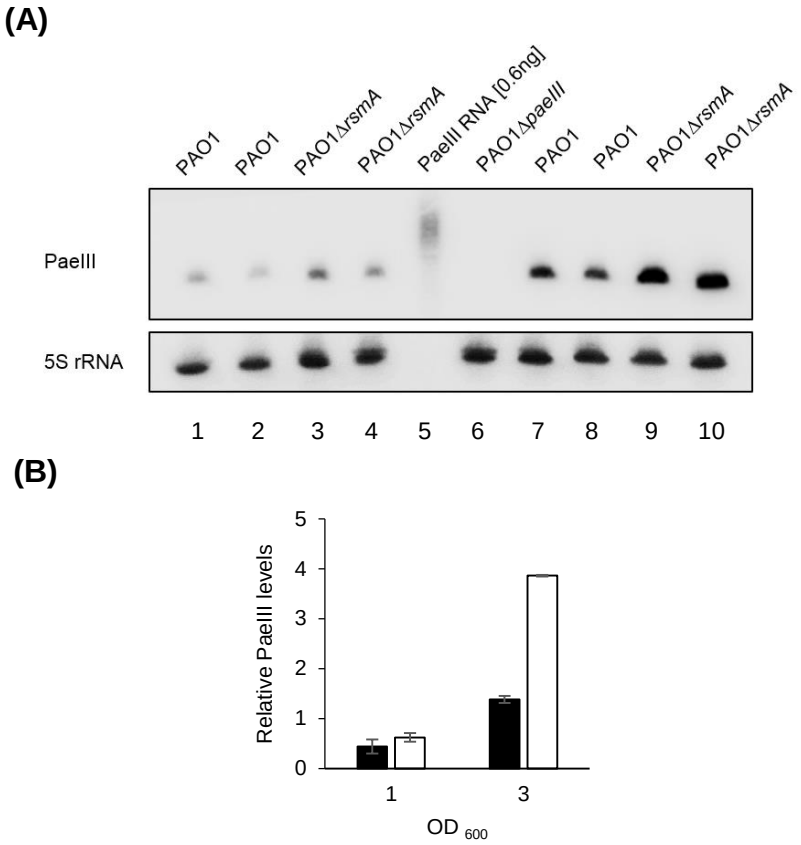


Figure 23. *RsmA* deletion enhances the levels of PaeIII.

(A) PAO1, PAO1Δ*rsmA* and PAO1Δ*paeIII* were grown in LB medium with 0.1% Triton X-100. Triton X-100 was supplemented to prevent cell aggregation in the PAO1Δ*rsmA* strain. The cells were harvested at an OD_{600} of 1 and 3. The PaeIII levels were determined by Northern-blot analysis using the ^{32}P 5'- end labelled oligonucleotides W28 (PaeIII) and I26 (5S rRNA; loading control). Total RNA prepared from PAO1Δ*paeIII* (lane 6) and *in vitro* transcribed PaeIII RNA (lane 5; M31 and O29 were used for amplification; Table 5) were used as controls. Total RNA was prepared from two biological replicates of PAO1 (lanes 1, 2 (OD_{600} of 1) and 7, 8 (OD_{600} of 3)) and from two biological replicates of PAO1Δ*rsmA* (lanes 3, 4 (OD_{600} of 1) and 9, 10 (OD_{600} of 3)). **(B)** Relative PaeIII levels were quantified with ImageQuant Software (Molecular Dynamics) in PAO1 (black bars) and PAO1Δ*rsmA* (white bars). The error bars represent standard deviations for two biological replicates.

transcriptional level. That is why Sorger-Domenigg (2010) proposed that a transcriptional regulator, which might have a positive impact on *paeIII* transcription, is repressed by RsmA in the wild-type strain. A Northern-blot anaylsis was performed to verify the enhanced PaeIII abundance in the PAO1Δ*rsmA* strain. Indeed, a 2.5-fold up-regulation could be confirmed at an OD_{600} of 3. At an OD_{600} of 1 this up-regulation was less pronounced (Figure 23A and B).

4.2.2 Growth in stationary phase and at elevated temperatures

As described by Sorger-Domenigg (2010), PaeIII was 2.5-fold more abundant in stationary phase (OD_{600} of 3) when compared with exponential growth (OD_{600} of 1) in LB medium. Furthermore, the PaeIII levels were 1.5-fold elevated when the cells were grown at 43°C (Sorger-Domenigg, 2010). This increased PaeIII abundance might imply a function during heat shock and in stationary phase. Therefore, it was determined whether PaeIII is required for cell growth and viability under these conditions. The cells were diluted to an initial OD_{600} of 0.1 in LB medium supplemented with 0.1% Triton X-100, and growth was monitored for seven hours. Samples were taken at an OD_{600} of 2.5, 3 and 3.7 to prepare serial dilutions for the CFU determinations. As shown in Figure 24A and B, there was no difference concerning growth in stationary phase between PAO1 and PAO1Δ*paeIII*. Next, the growth of PAO1 and PAO1Δ*paeIII* was examined at elevated temperatures. The cells were grown to an OD_{600} of 1 at 37°C and were then transferred

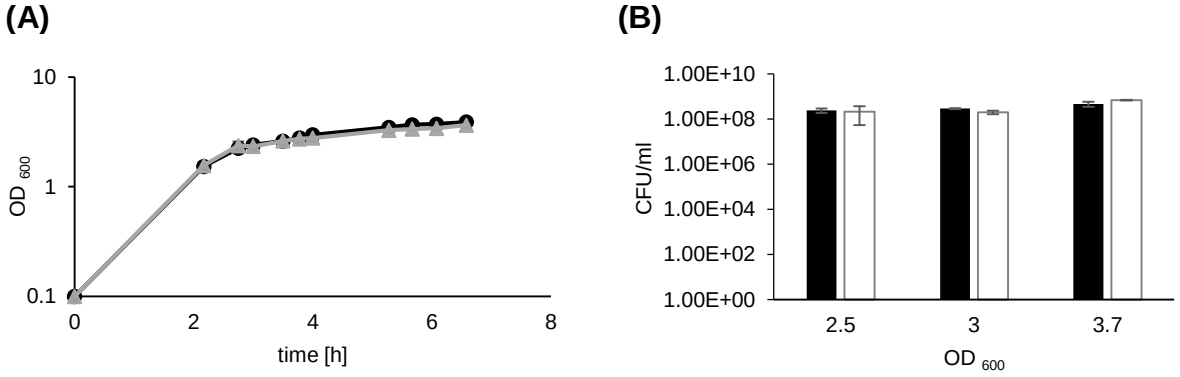


Figure 24. PaeIII is not required for growth in stationary phase.

(A) Growth of PAO1 (black dots) and PAO1Δ*paeIII* (grey triangles) in LB medium supplemented with 0.1% Triton X-100 was monitored for seven hours by measuring the optical density at 600 nm. Triton X-100 was added to prevent cell aggregation. **(B)** Samples of PAO1 (black bars) and PAO1Δ*paeIII* (white bars) were withdrawn at an OD_{600} of 2.5, 3 and 3.7. Serial dilutions from 10^{-1} to 10^{-8} were prepared and 10 µl of these dilutions were spotted on dried LB plates, which were incubated overnight at 37°C. The error bars represent standard deviations for two biological replicates.

to 43°C. Samples for the CFU determinations were taken 15, 45 and 75 minutes thereafter. No reduced growth rate (Figure 25A) or cell viability (Figure 25B) was observed for PAO1Δ*paeIII* compared with PAO1 when the cells were exposed to 43°C. Therefore, PaeIII appears to be non-essential for growth at elevated temperatures.

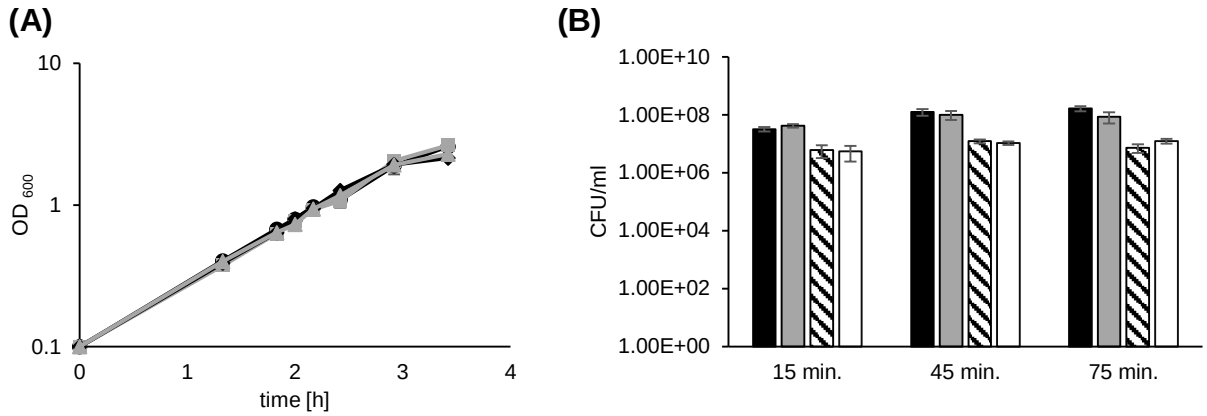


Figure 25. PaeIII is not required during growth at 43°C.

(A) PAO1 and PAO1Δ*paeIII* were grown in LB medium to an OD_{600} of 1, then PAO1 (black dots) and PAO1Δ*paeIII* (grey squares) were transferred to 43°C, while PAO1 (black diamonds) and PAO1Δ*paeIII* (grey triangles) remained at 37°C. **(B)** Samples were taken for CFU determination of PAO1 (black bars) and PAO1Δ*paeIII* (grey bars) after 15, 45 and 75 minutes of growth at 43°C and of PAO1 (tilted striped bars) and PAO1Δ*paeIII* (white bars) after 15, 45 and 75 minutes of growth at 37°C. The error bars represent standard deviations for two biological replicates.

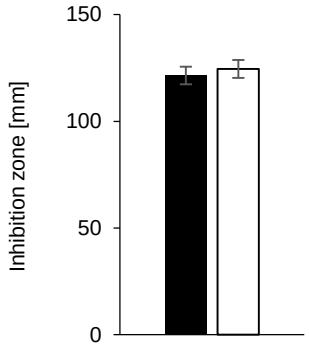
4.2.3 PaeIII does not enhance the resistance to tetracycline

A microarray analysis performed by Sorger-Domenigg (2010) revealed that the transcription of genes encoding the MexX/Y multidrug efflux pump was up-regulated in the *paeIII* over-expression strain PAO1(pJT-*PaeIII*) when compared with the control strain PAO1-(pJT19). The MexX/Y multidrug efflux pump is involved in the export of antibiotics like macrolides, aminoglycosides and tetracyclines (Masuda *et al.*, 2000; Jeannot *et al.*, 2005). This contributes significantly to the resistance to this classes of antibiotics (Poole, 2004). Sorger-Domenigg (2010) reported that PAO1(pME-*PaeIII*) was more resistant to tetracycline than the wild-type strain and the *paeIII* deletion mutant. Furthermore, PAO1Δ*paeIII*(pME4510) was less resistant to tetracycline than PAO1(pME4510).

To verify this increased antibiotic resistance in the presence of PaeIII, antibiotic susceptibility tests were performed. Disc diffusion assays (discs and strips) were used to determine the tetracycline susceptibility of PAO1 and PAO1Δ*paeIII* (3.10). The cells were grown in LB medium before applying the tetracycline test discs (30 µg/ml). The inhibition

zone observed in both strains was ~ 125 mm (Figure 26 A), indicating that the susceptibility to tetracycline of the *paeIII* mutant is the same as for the wild-type strain. In addition, minimum inhibitory concentration evaluator strips were employed. The same minimal in hibitory concentration (MIC) for tetracycline was observed for PAO1 and PAO1Δ*paeIII* with MIC evaluator strips (Figure 26B). Moreover, the susceptibility of a *paeIII* over-expression strain to tetracycline, measured with MIC evaluator strips, was not reduced when compared with the empty plasmid control (Figure 26C). In conclusion, these results suggest that PaeIII does not influence the resistance to tetracycline.

(A)



(B)

Strain	MIC [µg/ml]
PAO1	32
PAO1Δ*paeIII*	32

(C)

Strain	MIC [µg/ml]
PAO1(pME4510)	32 – 64
PAO1Δ*paeIII*(pME4510)	32 – 64
PAO1(pME4510*paeIII*)	32 – 64
PAO1Δ*paeIII*(pME4510*paeIII*)	32 – 64

Figure 26. PaeIII does not enhance the resistance to tetracycline.

(A) The growth inhibition zone of PAO1 (black bar) and PAO1Δ*paeIII* (white bar) on LB plates was determined using tetracycline antimicrobial susceptibility test discs. Cultivation was performed in LB medium until the cells reached an OD_{600} of 2. The cells were plated on LB plates and tetracycline antimicrobial susceptibility test discs (30 µg/ml) were placed on the dried plates and incubated overnight at 37°C. The error bars represent standard deviations for two biological replicates. **(B)** PAO1 and PAO1Δ*paeIII* were grown in LB medium to an OD_{600} of 2, before the cells were spread on LB plates and minimum inhibitory concentration evaluator strips (0.015 µg/ml - 256 µg/ml tetracycline) were placed on the dried plates and incubated overnight at 37°C. **(C)** The tetracycline susceptibility of PAO1(pME4510), PAO1Δ*paeIII*(pME4510), PAO1(pME4510*paeIII*) and PAO1Δ*paeIII*(pME4510*paeIII*) was determined with the same minimum inhibitory concentration evaluator strips as in **(B)**.

4.2.4 RpoS is not reduced upon *paeIII* over-expression

A transcriptome analysis performed by Sorger-Domenigg (2010) revealed that transcription of the gene encoding the alternative sigma factor RpoS is 2.9-fold down-regulated upon *paeIII* over-expression. An interaction site of PaeIII with *rpoS* mRNA was predicted by Sorger-Domenigg (2010) with the program TargetRNA (Tjaden, 2008). The β-galactosidase values conferred by a plasmid encoded translational *rpoS::lacZ* fusion in the PAO1Δ*paeIII* strain were 2.5-fold enhanced in stationary phase when compared with the wild-type strain (Sorger-Domenigg, 2010).

Here, the RpoS levels were examined by quantitative Western-blotting to verify these findings. Therefore, a *paeIII* over-expression plasmid pME4510*tacpaeIII* harboring *paeIII* under the control of the *tac* promoter was constructed. *PaeIII* over-expression was induced with 1 mM IPTG and after 30 minutes the *paeIII* expression in strain PAO1-(pME4510 *tacpaeIII*) was higher than in the wild-type (Figure 27, lane 5). The Fluorescent Western-blotting technique (Odyssey Infrared Imaging System) was used to determine the RpoS levels in PAO1, PAO1Δ*paeIII*, PAO1(pME4510*tacpaeIII*), PAO1(pME4510*tac*) and PAO1Δ*rsmA*. As shown in Figure 28, the RpoS levels in PAO1Δ*paeIII* were comparable to those observed in the wild-type strain. Likewise, the RpoS levels in PAO1-(pME4510*tacpaeIII*) were indistinguishable from the control strain PAO1(pME4510*tac*) (Figure 28A). These results indicate that the RpoS levels are not affected by the presence

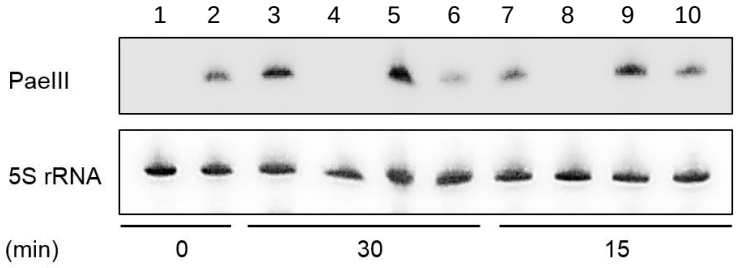


Figure 27. Verification of *paeIII* expression from plasmids.

PAO1 and PAO1Δ*paeIII* were grown in LB medium with 50 µg/ml gentamycin and were either transformed with pME4510*tac* or pME4510*tacpaeIII*. At an OD_{600} of 2.5, *paeIII* expression was induced with 1 mM IPTG and samples for RNA isolation were collected 15 and 30 minutes afterwards. The PaeIII levels were determined by Northern-blot analysis using the ^{32}P 5'- end labelled oligonucleotides W28 (PaeIII) and I26 (5S rRNA; loading control). Total RNA was prepared from the strains PAO1Δ*paeIII*(pME4510*tacpaeIII*) (lane 1) and PAO1(pME4510*tacpaeIII*) (lane 2) 0 minutes after induction with 1 mM IPTG (non-induced control). Total RNA was prepared from the strains PAO1-Δ*paeIII*(pME4510*tacpaeIII*) (lane 3), PAO1Δ*paeIII*(pME4510*tac*) (lane 4), PAO1(pME4510*tacpaeIII*) (lane 5) and PAO1(pME4510*tac*) (lane 6) 30 minutes after addition of 1 mM IPTG. Total RNA was prepared from the strains PAO1Δ*paeIII*(pME4510*tacpaeIII*) (lane 7), PAO1Δ*paeIII*(pME4510*tac*) (lane 8), PAO1(pME4510*tacpaeIII*) (lane 9) and PAO1(pME4510*tac*) (lane 10) 15 minutes after addition of 1 mM IPTG.

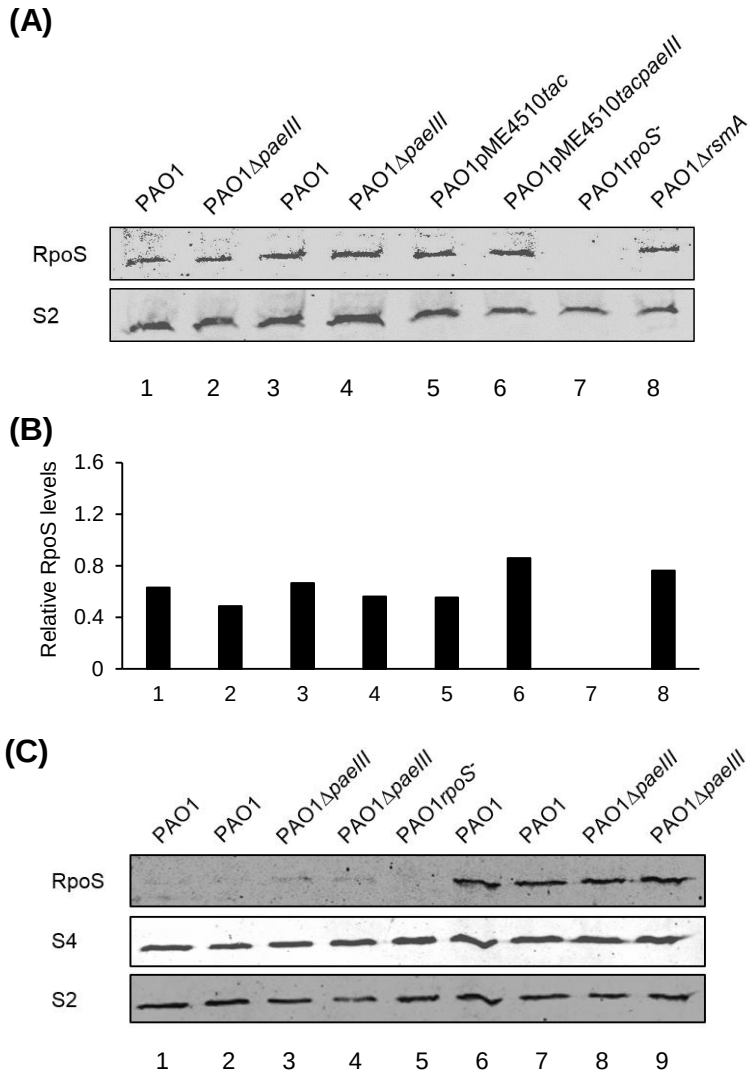


Figure 28. PaeIII has no influence on RpoS abundance.

(A) RpoS signals were detected by immunoblotting in PAO1, PAO1Δ*paeIII*, PAO1(pME4510*tac*), PAO1(pME4510-*tacpaeIII*), PAO1*rpoS*- and PAO1Δ*rsmA*. PAO1(pME4510*tac*) and PAO1(pME4510*tacpaeIII*) were grown in LB medium and *paeIII* over-expression was induced with 1 mM IPTG at an OD_{600} of 2 and the cells were harvested at an OD_{600} of 2.5. The strains PAO1, PAO1Δ*paeIII*, PAO1*rpoS*- and PAO1Δ*rsmA* were grown in LB medium and samples were collected at an OD_{600} of 2.5. Cell lysates prepared from two biological replicates of PAO1 (lanes 1 and 3) and PAO1Δ*paeIII* (lanes 2 and 4) and from PAO1(pME4510*tac*) (lane 5), PAO1(pME4510*tacpaeIII*) (lane 6), PAO1*rpoS*- (lane 7) and PAO1Δ*rsmA* (lane 8) were used for quantitative Western-blotting. The blots were probed with anti-RpoS antibodies (dilution 1: 10,000; Table 6). Ribosomal protein S2 (dilution 1: 10,000; Table 6) was used as a loading control. The antibody-antigen complex was visualized with the Rockland IRDye 800 conjugated anti-goat or anti-rabbit IgG (dilution 1: 15,000; Table 6) using the Odyssey Infrared Imaging system. **(B)** Quantification of the Western-blot results shown in **(A)**, the numbers correspond to the lanes above. The relative RpoS levels were calculated with ImageQuant software (Molecular Dynamics). **(C)** PAO1, PAO1Δ*paeIII* and PAO1*rpoS*- were cultivated in LB medium and harvested at an OD_{600} of 1 and 3. Cell lysates prepared from two biological replicates of PAO1 (lanes 1, 2 (OD_{600} of 1) and 6, 7 (OD_{600} of 3)) and PAO1Δ*paeIII* (lanes 3, 4 (OD_{600} of 1) and 8, 9 (OD_{600} of 3)) and from PAO1*rpoS*- (lane 5) were used for quantitative Western-blotting. The abundance of the RpoS protein was visualized by immunoblotting with anti-RpoS antibodies. The ribosomal proteins S2 (dilution 1: 1,000) and S4 (dilution 1: 1,000; Table 6) were used as loading controls. Signal detection was achieved with the Odyssey Infrared Imaging system.

or absence of PaeIII. In addition, the abundance of RpoS in a PAO1Δ*rsmA* strain was determined to investigate if RsmA is involved in *rpoS* regulation. In *Pseudomonas fluorescens* CHA0, it was shown that *rpoS* is under negative control of RsmA (Heeb *et al.*, 2005). However, this seems not to be the case in *P. aeruginosa,* where the RpoS levels of the wild-type strain were comparable to those observed in the PAO1Δ*rsmA* strain (Figure 28A).

4.2.5 No phenotypical evidence for PaeIII-mediated regulation of *ladS*

The lost adherence sensor LadS is a hybrid sensor kinase of the Gac/Rsm two-component system. A putative interaction site of *ladS* mRNA with PaeIII was identified by Sorger-Domenigg (2010) with the software TargetRNA (Tjaden, 2008). LadS positively controls biofilm formation and secondary metabolism (Figure 10) (Ventre *et al.*, 2006). The hypothesis that PaeIII might negatively regulate *ladS* is supported by the observation that many transcripts, which were up-regulated in strain PAO1(pJT*paeIII*) (Sorger-Domenigg, 2010), were likewise increased in a PAK *ladS* deletion strain (Ventre *et al.*, 2006). LadS affects *hcnA* expression, biofilm formation and RsmY abundance by indirectly stimulating the GacS function (Chambonnier *et al.*, 2016). To obtain further evidence for a PaeIII-mediated regulation of *ladS*, *hcnA* expression, biofilm formation and RsmY production were assessed in the *paeIII* deletion strain.

4.2.5.1 The expression of *hcnA* is not altered in the absence of PaeIII

The cyanide biosynthesis operon *hcnABC* is controlled by the Gac/Rsm regulatory network (Pessi and Haas, 2000). The negative regulation of the *hcn* gene cluster by RsmA occurs at the post-transcriptional level (Pessi *et al.*, 2001) and is suppressed by the RsmA binding RNAs RsmY/Z. Reduced *rsmY/Z* expression leads to reduced RsmA titration and consequently to increased *hcnA* repression (Figure 10) (Pessi *et al.*, 2001). The β-galactosidase values conferred by a plasmid encoded translational *hcnA::lacZ* fusion were therefore determined in the presence and absence of PaeIII. The strains PAO1Δ*gacA* and PAO1Δ*rsmA* were used as controls. The strains PAO1, PAO1Δ*paeIII*, PAO1Δ*gacA* and PAO1Δ*rsmA*, respectively, were transformed with plasmid pME3843 harboring a translational *hcnA::lacZ* fusion (Table 4). The cells were grown in LB medium supplemented with 0.1% Triton X-100 to an OD_{600} of 2.2. As expected, *hcnA* translation was ~ 4-fold enhanced in the PAO1Δ*rsmA* strain when compared with the wild-type strain. HcnA-LacZ synthesis was reduced in the *gacA* mutant strain by 50% when compared with the PAO1 strain (Figure 29). Nevertheless, *hcnA::lacZ* translation was comparable

in PAO1 and PAO1Δ*paeIII* (Figure 29). This led to the conclusion that *paeIII* deletion does not impact on *hcnA* expression, which in turn indicated that PaeIII does not affect *ladS* expression.

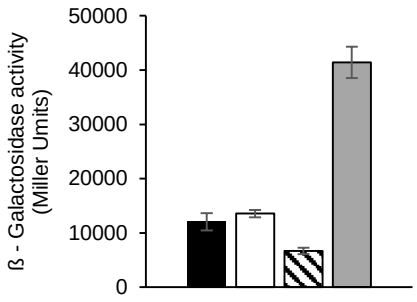


Figure 29. PaeIII does not affect *hcnA* expression.

PAO1(pME3843) (black bar), PAO1Δ*paeIII*(pME3843) (white bar), PAO1Δ*gacA*(pME3843) (tilted striped bar) and PAO1Δ*rsmA*(pME3843) (grey bar) were grown in LB medium supplemented with 0.1% Triton X-100 to an OD_{600} of 2.2. The bars represent the β-galactosidase values conferred by the plasmid pME3843 encoded translational p*tac-hcnA::lacZ* fusion. The error bars represent standard deviations for three biological replicates.

4.2.5.2 Biofilm formation is not altered in PAO1Δ*paeIII*

Both LadS and GacS have a positive effect on the expression of *rsmY* and *rsmZ* (Babitzke and Romeo, 2007). As a potential regulation of the *ladS* mRNA by PaeIII may have an indirect impact on biofilm formation, this was assessed in PAO1 and PAO1Δ*paeIII* using a crystal violet assay. As shown in Figure 30, there are no significant differences in biofilm

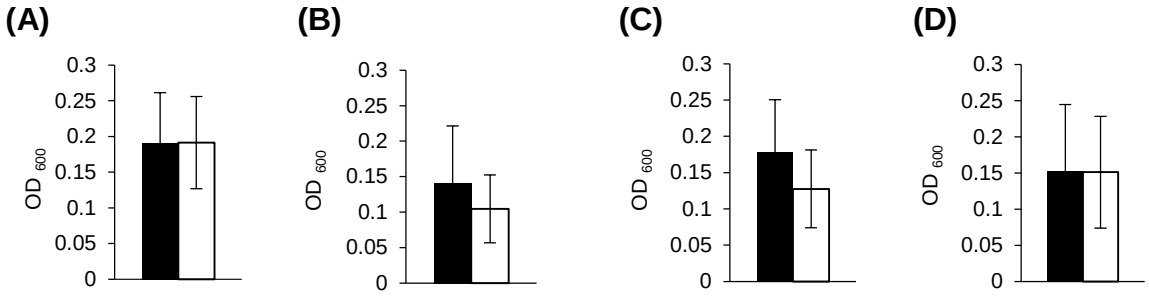


Figure 30. Biofilm formation is not altered in PAO1Δ*paeIII*.

Biofilm formation was quantified using a static crystal violet assay as described by Merritt *et al.* (2005). **(A and B)** PAO1 (black bars) and PAO1Δ*paeIII* (white bars) were grown overnight in LB medium at 37°C. Then the cells were diluted to an initial OD_{600} of 0.05 and were further incubated under aerobic conditions for 24 hours **(A)** and 48 hours **(B)** using 96-well plates. **(C and D)** PAO1 (black bars) and PAO1Δ*paeIII* (white bars) were incubated under anaerobic conditions for 24 hours **(C)** and 48 hours **(D)** using 96-well plates. To create an aneraerobic environment, the cells were cultivated in an anaerobic jar with gas packs and 100 mM KNO_3 was added to the LB medium. The error bars represent standard deviations for six biological replicates.

formation between the wild-type strain and the PAO1Δ*paeIII* strain under aerobic or anaerobic conditions. These results again argue against a role of PaeIII in *ladS* expression.

4.2.5.3 RsmY levels are unchanged in the absence of PaeIII

A negative post-transcriptional regulation of *ladS* exerted by PaeIII could result in altered LadS levels and therefore in decreased RsmY and RsmZ levels. RsmY was chosen for this experiment because the abundance of the sRNA is constant throughout growth, unlike RsmZ, which is predominantly expressed in stationary phase (Kay *et al.*, 2006). As shown in Figure 31B, the normalized levels of RsmY in the wild-type strain are indistinguishable from the abundance in a *paeIII* mutant strain. Therefore, PaeIII has no indirect effect on RsmY abundance under the observed conditions. These data are in line with the previous results concerning *hcnA* expression (4.2.5.1) and biofilm formation (4.2.5.2). This leads to the conclusion that there is no evidence to corroborate the hypothesis that PaeIII regulates *ladS*.

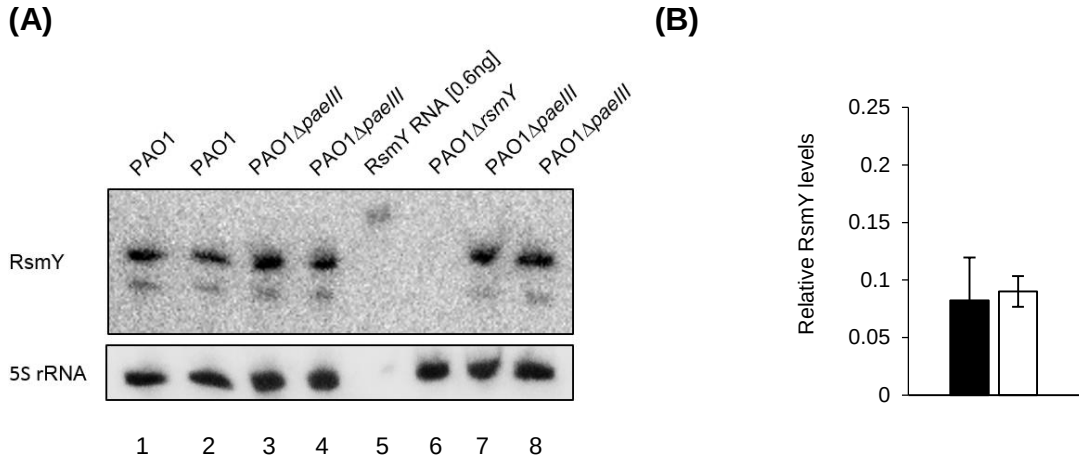


Figure 31. RsmY levels are unchanged in PAO1Δ*paeIII*.

(A) PAO1, PAO1Δ*paeIII* and PAO1Δ*rsmY* were grown in LB medium to an OD_{600} of 3. The RsmY levels were determined by Northern-blot analysis using the ^{32}P 5'- end labelled oligonucleotides T28 (RsmY) and I26 (5S rRNA; loading control). Total RNA was prepared from two biological replicates of PAO1 (lanes 1 and 2) and four biological replicates of PAO1Δ*paeIII* (lanes 3, 4, 7 and 8). Total RNA prepared from PAO1Δ*rsmY* cells (lane 6) and *in vitro* transcribed RsmY RNA (lane 5; U26 and V26 was used for amplification; Table 5) were used as controls. 5S rRNA served as a loading control. **(B)** The relative RsmY levels determined in PAO1 (black bar) and PAO1Δ*paeIII* (white bar) by Northern-blot analysis were quantified with the program ImageQuant (Molecular Dynamics). The error bars represent standard deviations for two biological replicates (PAO1) and for four biological replicates (PAO1Δ*paeIII*), respectively.

5 Discussion

5.1 PaeII

5.1.1 Expression of *paeII*

The small regulatory RNA PaeII is highly expressed in minimal medium, like BM2 or M9 supplemented with various carbon sources, but not in LB medium. According to the Northern-blot analysis (Figure 13), PaeII reaches a total of 1,400 molecules/cell in BM2 minimal medium. In general, the levels reported so far for other small RNAs are variable, ranging from 100 to 5000 copies per cell (Altuvia *et al.*, 1997; Hnilicova *et al.*, 2014).

Since PaeII is induced in minimal media, NtrC, which regulates nitrogen assimilation (Li and Lu, 2007), was a possible candidate for *paeII* regulation. However, as shown in the Northern-blot analyses (Figure 15) neither CbrB nor NtrC are required for *paeII* transcription. Nevertheless, there are several other transcriptional regulators that may be potential candidates (Table 1). Most of the known enhancer-binding proteins in *P. aeruginosa* regulate gene expression as a response to environmental cues. Frequently, they act as response regulators of a two-component system. Nevertheless, several EBPs like the NtrC family proteins DmpR and XylR are not part of a two-component system (Shingler *et al.*, 1993). These transcriptional regulators directly respond to the presence of aromatic compounds without a sensory protein. DmpR and XylR specifically activate the transcription of genes necessary for the degradation of these compounds (Shingler and Moore, 1994). DmpR controls the catabolism of phenols and methylphenols (Shingler *et al.*, 1993), whereas XylR controls the degradation of toluenes and xylenes (Inouye *et al.*, 1987). On the other hand, the EBP PhhR activates transcription of the *phh* operon and is required for growth in media with L-phenylalanine or L-tyrosine as the sole carbon source. However, none of these substrates is present in BM2 medium, which makes it unlikey that one of these regulators is involved in *paeII* transcription.

5.1.2 *RpoN* is not regulated by PaeII

Amongst many other functions, the alternative sigma factor RpoN regulates nitrogen assimilation genes (Hirschman *et al.*, 1985; Gussin *et al.*, 1986). Therefore, it was speculated that PaeII might be involved in nitrogen metabolism. However, the results of growth assays did not substantiate this hypothesis. The growth of a *paeII* deletion mutant was indistinguishable from the wild-type strain in nitrogen depleted medium (Figure 16A and

B). Another possibility was that a feedback loop exists, where PaeII controls its own expression by modulating the *rpoN* mRNA levels. This kind of autoregulatory feedback loop has been described in *E. coli* and *Salmonella enterica* for σ^{24} (RpoE)-regulated sRNAs. RybB and MicA are both transcribed from σ^{24}-dependent promoters and down-regulate their own expression by decreasing the RpoE activity (Papenfort *et al.*, 2006; Udekwu and Wagner, 2007; Thompson *et al.*, 2007). Transcriptional and translational fusions of *rpoN* to the *lacZ* reporter gene were employed to test this hypothesis. However, *rpoN-lacZ* transcription and *rpoN::lacZ* translation were similar in the presence and absence of PaeII, independently of the growth phase and the selected carbon source in minimal medium (Figure 22). These results seem to exlude the possibility that PaeII influences *rpoN* expression and its own transcription *via* a feedback loop.

5.1.3 *DksA2* is not regulated by PaeII

The zinc-independent transcription factor DksA2 was predicted to be a potential target of PaeII (Rindler 2014). Toluene-induced *paeII* expression in PAO1Δ*paeII*(pJT*paeII*) resulted in a 12.3-fold up-regulation of the *dksA2* transcript when compared with PAO1 Δ*paeII*(pJT19) (Rindler 2014). To verify these results, a Real-time quantitative PCR (RT-qPCR) was performed under the same growth conditions (Figure 19). The *dksA2* levels observed in PAO1 did not differ from those in the *paeII* mutant. Furthermore, the *dksA2* transcript abundance was indistinguishable in PAO1Δ*paeII*(pJT*paeII*) and PAO1Δ*paeII* (pJT19) (Figure 19). These findings led to the assumption that the *dksA2* transcript abundance is not influenced by the sRNA PaeII. To substantiate these results, reporter gene fusions were used to study the *dksA2* expression levels. The β-galactosidase values conferred by a transcriptional *dksA2-lacZ* fusion were in line with the RT-qPCR results (Figure 21C and D). In addition, *dksA2::lacZ* translation was similar in the presence and absence of PaeII in BM2 medium supplemented with succinate or mannitol (Figure 21A and B). Interestingly, the *dksA2* levels were elevated in an *rpoN* deletion strain (Figure 21A and B), which is in contrast to the expectations, as *dksA2* was thought to require σ^{54} for transcription (Figure 20). A direct or indirect negative regulation of RpoN on *dksA2* transcription might explain this observation. Sigma factors have been shown to modulate gene expression by activation, as well as by repression (Jishage *et al.*, 2002; Laurie *et al.*, 2003; Levi-Meyrueis *et al.*, 2015). The abundance of RpoD (σ^{70}) is higher than the amount of core RNAP in the cell, which creates a constant competition for holoenzyme formation between different sigma factors (Osterberg *et al.*, 2011; Feklistov *et al.*, 2014). This form of passive regulation has been described for σ^{32} (RpoH) and σ^{54} (RpoN) (Jishage *et al.*, 2002; Laurie *et al.*, 2003). Apart from this, two other models of negative regulation by σ^{38} (RpoS) have been proposed by Levi-Meyrueis *et al.* (2015) in *Salmonella*. The first model includes a direct form of repression, mediated by binding of RNAPσ^{38}, which impedes

transcription initiation with other holoenzymes. This leads to promoter occlusion and transcriptional interference. Maybe this mode of action applies to RpoN as well and a similar mechanism might be operative at the *dksA2* promoter, which contains a putative σ^{70}- and σ^{54}-binding region (Figure 20). This might explain the high transcription levels observed with a *dksA2-lacZ* fusion in an *rpoN* mutant, where this kind of repression would be relieved. The second model specifies that repression involves effector molecules (Levi-Meyrueis *et al.*, 2015), which has also been proposed for σ^{54} in *P. aeruginosa* (Sana *et al.*, 2013). It could be possible that RpoN, together with an EBP, activates an unknown repressor of *dksA2* transcription. This could also result in elevated *dksA2* expression upon *rpoN* deletion.

5.1.4 PaeII is not required during zinc depletion

Many Zur-regulated genes were found to be down-regulated upon *paeII* over-expression (Rindler, 2014). One possibility was that PaeII itself binds to the zinc uptake regulator Zur. However, electrophoretic mobility shift assays (EMSA) did not support this hypothesis (E. Sonnleitner, unpublished data). Therefore, the general impact of PaeII on growth rates and cell viability during zinc depletion was determined. As shown in Figure 17 and Figure 18, cell growth and viability were not altered in PAO1Δ*paeII* when compared with the wild-type strain. In general, growth was impaired to a similar extent in the wild-type and the *paeII* mutant at low zinc concentrations. The absence of a growth defect of the *paeII* mutant does not exclude the possibility that PaeII is involved in zinc homeostasis. Hence, a PAO1 *znuA* mutant displayed no growth defect relative to the wild-type strain upon growth in medium supplemented with 60 µM TPEN (Pederick *et al.*, 2015). ZnuA is a high affinity zinc-binding protein that delivers Zn^{2+} to the ZnuABC transporter (Figure 4), and loss of *znuA* resulted in a 60% reduction of the intracellular zinc concentration (Pederick *et al.*, 2015). Although the enhanced *dksA2* levels observed in the transcriptome analysis (Rindler, 2014) could not be supported, it is remarkable that 34 out of 44 genes up-regulated more than 4-fold in PAO1Δ*znuA* (Pederick *et al.*, 2015) were likewise up-regulated upon over-expression of *paeII*. Thus, elevated PaeII levels affect the transcriptome in a similar way as the absence of *znuA*. A *znuA* deletion strain experiences zinc depletion due to reduced Zn^{2+}-binding abilities. Taking this into account, it is tempting to speculate that *znuA* might be directly down-regulated by PaeII. A subtle negative effect of PaeII on *znuA* translation might be an additional regulatory element to prevent zinc accumulation, which can be toxic for the cell (Silver, 1996; Bruins *et al.*, 2000; McDevitt *et al.*, 2011). Morevoer, a putative interaction site with PaeII was detected upstream of the *znuA* mRNA (Supplementary Figure S3). Nevertheless, it still remains elusive why the *dksA2* transcript levels are not up-regulated in the presence of PaeII (Figure 19 and Figure 21). They were expected to increase upon *paeII* over-expression, even though this could be indirect *via*

reduced zinc availability. The *znuA* levels themselves were not altered upon *paeII* over-expression in the RNA-seq analysis, but it is conceivable that an inhibitory effect on translation is not manifested by sRNA-mRNA target degradation, so that the transcript levels remain unchanged (Caron *et al.*, 2010). Taken together, it seems worth to investigate a putative effect of PaeII on *znuA*. In addition, the Zn^{2+} levels of a *paeII* over-expression strain should be compared with the PAO1Δ*paeII* strain and the wild-type strain, to verify if zinc abundance is decreased in the presence of PaeII. This could be achieved with a whole cell metal accumulation analysis using inductively coupled plasma-mass spectrometry (ICP-MS) (Pederick *et al.*, 2015).

5.2 PaeIII

5.2.1 Expression of *paeIII*

The sRNA PaeIII is transcribed from a σ^{70}-dependent promoter and was detected in LB, SCFM (Sorger-Domenigg, 2010) and BM2-succinate medium (Supplementary Figure S3). The highest levels were reached in LB medium upon entry into stationary phase, which is why in this study a possible function of PaeIII in the regulation of the transition from exponential to stationary phase was analyzed. The growth and viability of the *paeIII* deletion mutant was similar when compared with the wild-type strain (Figure 24A and B). As *paeIII* transcription was enhanced at elevated temperatures (Sorger-Domenigg, 2010), cells grown at 37°C were compared with cultures exposed to 43°C. Cell growth and viability at elevated temperatures were comparable in strains PAO1 and PAO1 Δ*paeIII* (Figure 25A and B). These results indicated that PaeIII is not essential for growth at 43°C or for transition from exponential to stationary phase. However, it is conceivable that the increased PaeIII abundance under heat shock conditions is more pronounced at higher temperatures. Even temperatures as high as 50°C may be applicable, because 60% of PAO1 cells were able to survive eight minutes of exposure to 50°C (Suh *et al.*, 1999).

When Sorger-Domenigg (2010) determined the PaeIII steady state levels in different PAO1 mutant strains by Northern-blotting, a 2-fold increase was found in a *rsmA* mutant strain at an OD_{600} of 3, an observation that was confirmed (Figure 23). The RNA-binding protein RsmA is negatively regulated by the GacS/GacA two-component system. The two GacS-regulated sRNAs RsmY and RsmZ sequester RsmA and alleviate the repressive function of RsmA on target mRNAs (Sonnleitner and Haas, 2011). Sorger-Domenigg (2010) hypothesized that this observation could be due to RsmA-mediated down-regulation of a transcriptional regulator that affects *paeIII* expression. A search within the RsmA regulon (Burrowes *et al.*, 2006) for regulatory factors that are up-regulated in the *rsmA*

mutant was conducted. Two putative transcriptional regulators were identified: PA2547 (3.4-fold up-regulated) and PA3269 (2.1-fold up-regulated) (Winsor *et al.*, 2016). Their functions are not yet characterized, but their enhanced expression in the *rsmA* mutant could be in line with a stimulatory effect on PaeIII. Therefore, it remains to be investigated whether these factors impact on *paeIII* transcription.

5.2.2 PaeIII does not enhance the resistance to tetracycline

Transcriptomics (Sorger-Domenigg, 2010) revealed that the transcript levels of mRNAs encoding the MexX/Y multidrug efflux pump were up-regulated upon *paeIII* over-expression. MexX/Y is involved in the import of antibiotics like macrolides, aminoglycosides and tetracyclines (Masuda *et al.*, 2000; Jeannot *et al.*, 2005). Sorger-Domenigg (2010) reported an increased tetracycline resistance in a *paeIII* over-expression strain. However, these results could not be verified in antibiotic susceptibility tests (Figure 26).

5.2.3 RpoS is not reduced upon *paeIII* over-expression

The *rpoS* mRNA encoding σ^{38} was predicted to be a target of PaeIII. Evidence for this hypothesis has been provided by Sorger-Domenigg (2010) and Rindler (2014): a microarray analysis showed that *paeIII* over-expression led to down-regulation of *rpoS* (2.9-fold). As the webtool TargetRNA (Tjaden, 2008) predicted an interaction site with the Shine-Dalgarno sequence of *rpoS* mRNA, the β-galactosidase values conferred by a translational *rpoS::lacZ* fusion were determinded (Sorger-Domenigg, 2010). The *rpoS* levels were 2-fold higher in a *paeIII* mutant when compared with the wild-type strain, which indicated that PaeIII affects *rpoS* translation. However, it was not possible to introduce mutations in the putative interaction site of *rpoS* mRNA with PaeIII, as this region comprises the SD sequence of *rpoS*. Therefore, an influence of PaeIII on *rpoS* translation could not be verified (Rindler, 2014). Here, it was investigated whether a regulatory role of PaeIII on *rpoS* mRNA could be observed at the protein level. The amount of RpoS synthesis was determined by Western-blot analysis. However, the RpoS abundance was not increased in the *paeIII* mutant strain at an OD_{600} of 1, 2.5 or 3 when compared with the wild-type strain (Figure 28A, B and C). In addition, the RpoS levels were comparable in PAO1(pME4510*tacpaeIII*) (Figure 28A, lane 5) and the control strain PAO1(pME 4510*tac*) (Figure 28A, lane 6). Hence, the assumption that *rpoS* mRNA is regulated by PaeIII could not be corroborated. *RpoS* regulation involves several transcriptional and post-transciptional control elements (Figure 4) (Venturi, 2003). The regulatory mechanisms involved seem to work differently in some species. Whistler *et al.* (1998) and Heeb *et al.* (2005) reported that in *P. fluorescens*, the Gac/Rsm signal transduction pathway

positively influences RpoS synthesis, whereas RsmA negatively regulates *rpoS* transcription. In contrast, RsmA was shown to act on *rpoS* in a positive manner in *Pseudomonas sp.M18* (Ge *et al.*, 2007). Apparently, neither mechanism seems to be operative in *P. aeruginosa*, because the RpoS levels in an *rsmA* mutant strain were the same as in the wild-type strain (Figure 28A, lane 8).

5.2.4 No phenotypical evidence for PaeIII-mediated regulation of *ladS*

The lost adherence sensor LadS was identified by Sorger-Domenigg (2010) as a potential target of PaeIII using the webtool TargetRNA (Tjaden, 2008). This protein is a hybrid sensor kinase of the GacS/GacA two-component system and enhances the GacS function (Ventre *et al.*, 2006). Thus, LadS promotes secondary metabolism, quorum sensing and biofilm formation (reviewed by Sonnleitner and Haas (2011)). The transcript levels of many LadS-regulated genes like *exoS*, *pscEFI*, *exsCEB*, *popB*, *pcrH* and *pscO* were increased upon *paeIII* over-expression (Sorger-Domenigg, 2010). LadS and GacS have a positive effect on the expression of *rsmY* and *rsmZ* (Babitzke and Romeo, 2007). Therefore, RsmY levels, biofilm formation and *hcnA* translation were assessed in PAO1Δ*paeIII* compared with the wild-type strain. However, biofilm formation (Figure 30), *hcnA* translation (Figure 29) and the abundance of the sRNA RsmY (Figure 31) were comparable in the strains PAO1 and PAO1Δ*paeIII*. It is possible that LadS was a false positive prediction of the webtool TargetRNA (Tjaden, 2008). This hypothesis is corroborated by the fact that it was not possible to reproduce the interaction with the follow-up version of the program, TargetRNA2 (Kery *et al.*, 2014). In addition, no change in the *ladS* mRNA levels were observed in the microarray data by Sorger-Domenigg (2010).

5.3 Finding targets and functions of regulatory RNAs

Deep sequencing analysis is one way to identify potential target genes of regulatory RNAs, but this method was shown to be biased by false positive results. Furthermore, it is not possible to discern direct targets from downstream affected transcripts (Sittka *et al.*, 2008). Another approach is the validation of predicted interactions one by one using reporter gene assays. This is a time-consuming attempt, which is why finding new ways to optimize computational target prediction is vital. A comparative analysis by Pain *et al.* (2015) confirmed that CopraRNA (Wright *et al.*, 2014; Wright *et al.*, 2013) is currently the best program for sRNA target prediction. This may be routed in the fact that it takes evolutionary sequence conservation and RNA accessibility into account (Wright *et al.*, 2013; Wright *et al.*, 2014). PaeIII is only conserved in *P. aeruginosa* strains and not among other Pseudomonads, therefore the identification of targets with CopraRNA might not be as reliable as for more conserved sRNAs. A refined transcriptome analysis seems to be the method of choice to identify PaeIII function(s), including a pulse expression experiment with subsequent RNA-seq analysis. This method is currently the standard process in target identification, as it provides increased sensitivity, a more dynamic range and reduced technical artifacts in comparison to microarray experiments (Barquist and Vogel, 2015). *PaeIII* is expressed under conditions associated with infection, like growth in SCFM medium or the addition of non-activated human serum to the cells (Sorger-Domenigg, 2010). The 'dual RNA-seq' technique might be a way to decipher a potential involvement of PaeIII in host infection scenarios. This method, developed by Westermann *et al.* (2012), allows the simultaneous analysis of bacterial and host transcriptomes. This could be a suitable procedure to unravel the function of PaeIII.

Small RNA tagging, affinity purification and subsequent analysis with RNA-seq and mass spectrometry (Said *et al.*, 2009; Lalaouna *et al.*, 2015) is a currently ongoing project to determine potential PaeII protein and mRNA targets. The implication of sophisticated new methods may significantly curtail target identification. Techniques like the CLASH assay (cross-linking, ligation, and sequencing of hybrids) provide an innovative high throughput method to identify RNA-RNA interactions (Kudla *et al.*, 2011). This is a large-scale approach to discover new binding partners for RNAs with uncharacterized functions. However, this method has been established in yeast and relies on binding and cross-linking of the RNA to ribonucleoproteins, which is why only RNA-RNA hybrids close to a ribonucleoprotein complex can be identified. Therefore, the CLASH protocol needs to be modified for use in bacteria.

Ribosome profiling is another method for sRNA target prediction, as it can be used together with RNA-seq. The combined utilization of both methods makes it possible to distinguish between transcriptional and translational regulation at the genome-wide level.

This approach can also be used to identify post-transcriptionally controlled sRNA targets, where the mRNA is not subject to degradation, and no change in mRNA levels occurs (Ingolia, 2014; Barquist and Vogel, 2015).

Indeed, it can be difficult to unravel distinct functions of sRNAs, because most often sRNA regulation is redundant and only fine-tunes gene expression instead of providing a stable 'yes or no' outcome (Barquist and Vogel, 2015). Moreover, new modes of action employed by regulatory RNAs are continuously discovered and some sRNAs can use several modes of action. Apparently, a single sRNA can apply different mechanisms to control expression of various targets. The *trans*-encoded *Vibrio harveyi* quorum regulatory RNA Qrr3 employs three different mechanisms of target repression with distinct regulation strength and also activates a target prior to sRNA degradation (Feng *et al.*, 2015).

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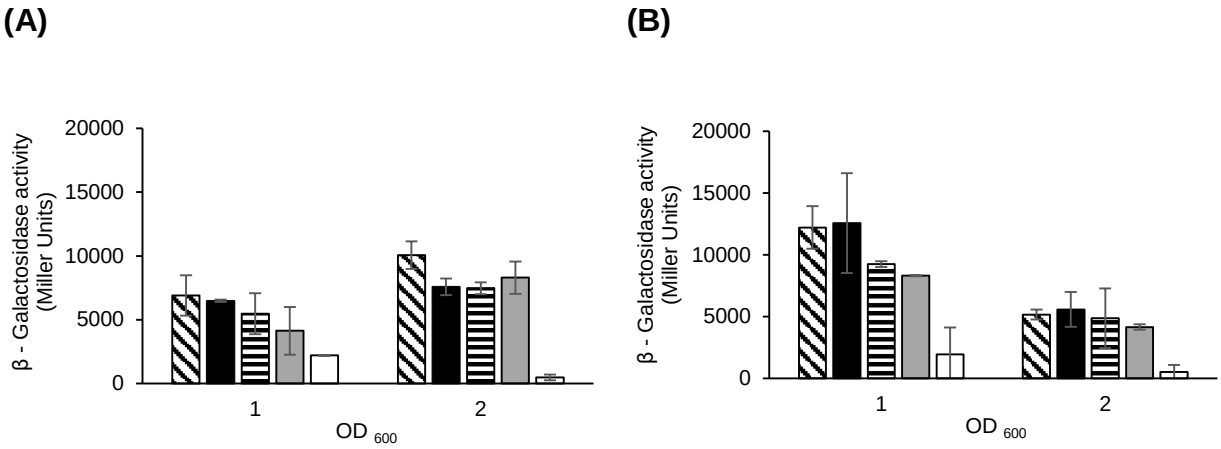
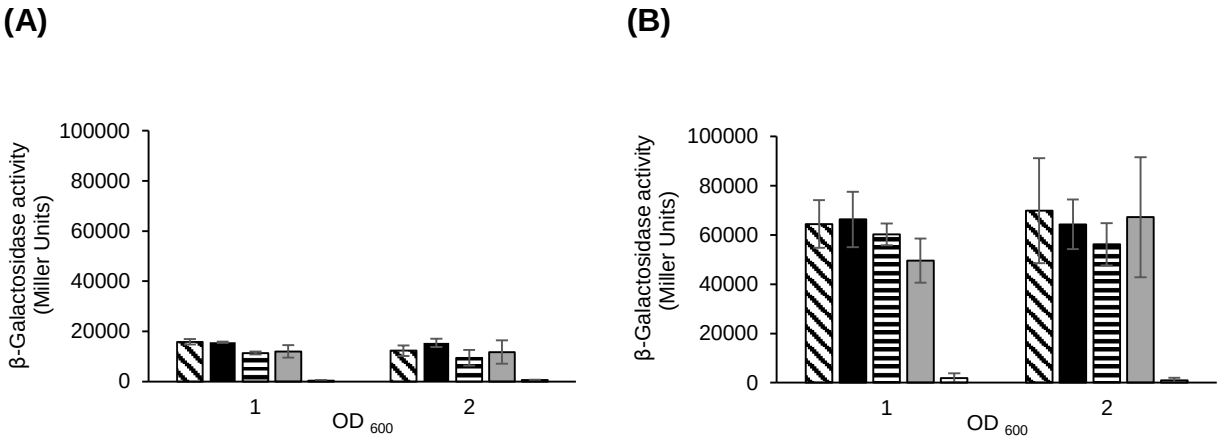
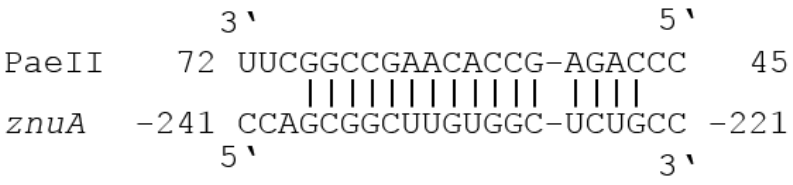
7 Supplementary Figures



Supplementary Figure S1. Transcription of *phrS* is stimulated by RpoN but not affected by PaeII.
(A and B) The strains PAO1(pME7233) (tilted striped bars), PAO1Δ*paeII*(pME7233) (black bars), PAO1(pME4510, pME7233) (horizontally striped bars), PAO1(pME4510*paeII,* pME7233) (grey bars) and PAO1Δ*rpoN*(pME7233) (white bars) were grown in BM2 minimal medium supplemented with 20 mM succinate **(A)** or 20 mM mannitol **(B)** to an OD_{600} of 1 and 2. The bars represent the β-galactosidase values conferred by the plasmid pME7233 encoded transcriptional *phrS-lacZ* fusion. The error bars represent standard deviations of two biological replicates.

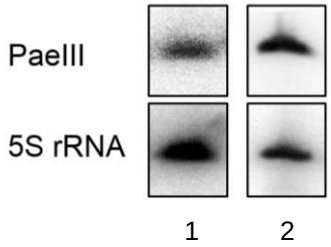


Supplementary Figure S2. Transcription of *crcZ* is activated by RpoN but not affected by PaeII.
(A and B) The strains PAO1(pME9806) (tilted striped bars), PAO1Δ*paeII*(pME9806) (black bars), PAO1(pME4510, pME9806) (horizontally striped bars), PAO1(pME4510*paeII*, pME9806) (grey bars) and PAO1Δ*rpoN*(pME9806) (white bars) were grown in BM2 minimal medium supplemented with 20 mM succinate **(A)** or with 20 mM mannitol **(B)** to an OD_{600} of 1 and 2. The bars represent the β-galactosidase values conferred by the plasmid pME9806 encoded transcriptional *crcZ-lacZ* fusion. The error bars represent standard deviations of two biological replicates.



Supplementary Figure S3. A possible interaction site of PaeII with *znuA* mRNA.

A putative interaction site of *znuA* and PaeII. Numbers mark the distance from the transcriptional start for *paeII* (+1) and the distance from the A (+1) of the start codon of *znuA*.



Supplementary Figure S4. Synthesis of PaeIII in BM2 medium.

Determination of the steady state levels of PaeIII by Northern-blot analysis. PAO1 was grown in BM2 medium supplemented with 20 mM succinate and 0.2 % L-glutamine to an OD_{600} of 1 and 2. 5S rRNA was used as a loading control. Total RNA was prepared from the strain PAO1 at an OD_{600} of 1 (lane 1) and at an OD_{600} of 2 (lane 2).

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11 Abstract

Pseudomonas aeruginosa is a human pathogen that causes nosocomial infections, especially in immunocompromised patients. Bacterial small RNAs are involved in regulating virulence, biofilm formation, uptake of various nutrients, membrane remodeling and many other processes. Although many *P. aeruginosa* sRNAs have been detected by computational prediction, their functions remain largely elusive. This work concentrated on the characterization and target identification of the sRNAs PaeII and PaeIII in the *P. aeruginosa* strain PAO1.

The sRNA PaeII is transcribed from a σ^{54}-dependent promoter and is highly abundant in minimal medium. The bacterial enhancer-binding protein (EBP) necessary for σ^{54}-dependent transcription of *paeII* was unknown. NtrC and CbrB were tested as potential candidates but both turned out to be not required for *paeII* transcription. The σ^{54}-dependent transcription of *paeII* led to the hypothesis that the sRNA might be involved in nitrogen metabolism. However, several growth assays did not corroborate this assumption. Although transcriptome analyses predicted a potential role for PaeII in zinc uptake and metabolism, several follow up studies did not support these predictions. For instance, a role of PaeII in the regulation of *dksA2* expression could not be verified.

The sRNA PaeIII is constitutively expressed and is most abundant in stationary phase as well as during growth at 43°C. Therefore, PaeIII was hypothesized to be required during transition from exponential to stationary phase or during growth at elevated temperatures. However, the results of growth assays did not support this assumption. A preceding study identified the mRNAs encoding the sensor kinase LadS and the sigma factor RpoS as potential targets of PaeIII. However, follow up experiments performed in this work did not substantiate these hypotheses.

12 Zusammenfassung

Pseudomonas aeruginosa ist ein humanpathogenes Bakterium, welches nosokomiale Infektionen, insbesondere bei immungeschwächten Patienten, auslösen kann. In Bakterien sind kleine RNAs (sRNAs) an Prozessen wie zum Beispiel Biofilmbildung, Virulenz, Nährstoffaufnahme oder Membranremodellierung beteiligt. Viele *P. aeruginosa* sRNAs wurden durch computergestützte Vorhersagen mithilfe bioinformatischer Programme entdeckt, jedoch ist die Funktion der meisten noch unbekannt. Diese Arbeit befasst sich mit der Charakterisierung der sRNAs PaeII und PaeIII in dem *P. aeruginosa* Stamm PAO1.

Die sRNA PaeII wird von einem σ^{54}-abhängigen Promotor (RpoN) transkribiert und ist in Minimalmedien in großen Mengen vorhanden. Das bakterielle Enhancer-bindende Protein (EBP), welches für die Transkription von *paeII* benötigt wird, ist bis dato unbekannt. NtrC und CbrB wurden als mögliche Kandidaten untersucht. Ein Einfluss beider Faktoren auf die *paeII* Transkription konnte jedoch nicht bestätigt werden. Da PaeII RpoN-abhängig transkribiert wird, wurde vermutet, dass diese sRNA eine Funktion im Stickstoffmetabolismus haben könnte. Vergleichende Analysen des Bakterienwachstums konnten dies aber nicht bestätigen. Obwohl Transkriptomanalysen eine Funktion von PaeII im Zinkmetabolismus vorhersagten, konnte in dieser Studie kein Hinweis für eine solche Beteiligung gefunden werden. So konnte zum Beispiel kein Einfluss von PaeII auf die Expression des Gens *dksA2*, das für einen Zink-unabhängigen Regulator kodiert, nachgewiesen werden.

PaeIII wird konstitutiv exprimiert und erreicht die höchste Konzentration in der stationären Phase und während des Wachstums bei 43°C. Die Hypothese, dass PaeIII unter diesen Bedingungen essentiell für das Wachstum von *P. aeruginosa* ist, konnte nicht bestätigt werden. Vorhergehende Studien indizierten, dass die Hybridsensorkinase LadS und der Sigmafaktor RpoS möglicherweise auf post-transkriptionaler Ebene von PaeIII reguliert werden. Die Ergebnisse dieser Arbeit konnten dies aber nicht bestätigen.