universität wien

DISSERTATION

Titel der Dissertation

„Novel *Pseudomonas aeruginosa* small regulatory RNAs“

Verfasserin

Mag. rer. nat. Theresa Sorger-Domenigg

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. nat.)

Wien, 2010

Studienkennzahl lt. Studienblatt:	A 091 441
Dissertationsgebiet lt. Studienblatt:	Genetik-Mikrobiologie
Betreuer:	Univ.-Prof. Dr. Udo Bläsi

INHALTSVERZEICHNIS

1 ZUSAMMENFASSUNG

Kleine regulatorische RNAs (sRNAs) spielen eine immer wichtigere Rolle, indem sie eine Vielzahl von physiologischen Prozessen modulieren. Die Gruppe der bakteriellen sRNAs beinhaltet mRNA leader Sequenzen, die die Genexpression in *cis* beeinflussen, sRNAs, die an Proteine binden oder Basenpaarungen mit RNA oder DNA eingehen. sRNAs kontrollieren die Expression von Virulenzgenen in pathogenen Bakterien, wie zum Beispiel in *Pseudomonas aeruginosa*, *Vibrio cholerae* und *Staphylococcus aureus*. Aufgrund neuer Detektionsmethoden, die das gesamte Genom untersuchen, wächst die Zahl von sRNAs stetig an, deren Funktionen hingegen bleiben weitgehend unbekannt und stellen den Gegenstand gegenwärtiger Untersuchungen dar.

Das Ziel meiner Arbeit war es neue unbekannte sRNAs in *P. aeruginosa* zu detektieren und ihre Funktion zu charakterisieren. Dafür wurde einerseits eine Methode zur direkten Klonierung von sRNAs (RNomics) verwendet und andererseits eine bioinformatische Methode, die sRNAs basierend auf der evolutionär konservierten RNA Struktur selektiert. Mithilfe von RNomics konnten drei neue sRNAs detektiert werden und mithilfe der Bioinformatik wurden sieben weitere sRNAs vorrausgesagt, von denen bei zwei die Expression experimentell nachgewiesen werden konnte. Weiters wurde eine alternative auf Bioinformatik basierte Suche nach potentiellen sRNAs angewendet, wobei folgende Suchkriterien verwendet wurden: intergene Regionen, 80-300 Nukleotide lange sRNAs, die von einem Promoter als auch einem Rho-unabhängigen Terminator flankiert werden. Das Ergebnis dieser Suche waren die drei sRNA Kandidaten PaeI, PaeII und PaeIII.

Die Synthese von PaeII wird von einem RpoN-abhängigen Promoter kontrolliert. PaeII wird gehäuft unter Nährstoff limitierenden Bedingungen exprimiert und hängt von der Guanosin Tetraphosphat Konzentration in der Bakterienzelle ab. Vergleichende Analysen des Transkriptoms des Wildtyp Stammes mit einer *paeII* Mutante zeigten, dass die mRNA

Konzentration einiger Gene, die für Proteine des Purin Biosynthese Apparates kodieren, in der Gegenwart von PaeII verringert ist. Es ist zu vermuten, dass die Purin Biosynthese unter Nährstoffmangel durch PaeII abgeschalten werden könnte, da sie zu diesem Zeitpunkt nicht benötigt wird.

PaeIII wird preferentiell während der stationären Wachstumsphase und in Medium, dem humanes Serum zugesetzt wurde, exprimiert. Dabei handelt es sich um Bedingungen, die denen während einer Infektion ähneln. Eine Untersuchung des Transkriptoms indizierte bei Überexpression von *paeIII* eine höhere Synthese von Komponenten der MexX/Y „Multidrug“ Effluxpumpe, die für den Transport einer Vielzahl von Antibiotika verwendet wird, sowie des Typ III Sekretionsapparates. Im Gegensatz dazu werden Gene, die in Pyocin- und Aminosäurebiosynthese und in anaerober Atmung involviert sind, bei *paeIII* Überexpression reprimiert, weiters scheint PaeIII die Translation von rpoS, das für σ^s kodiert, negativ zu regulieren.

Zusätzlich, wurde der Effekt von Hfq auf die *P. aeruginos*a sRNAs RsmY und RsmZ untersucht. Beide sRNAs binden an das Repressorprotein RsmA und verhindern, dass das Protein an mRNAs bindet und deren Translation blockiert. RsmA kontrolliert vor allem die Expression von Virulenzgenen. Vorangegangene Experimente bewiesen, dass Hfq an RsmY, aber nicht an RsmZ, bindet und es dadurch stabilisiert. Ich konnte experimentell zeigen, dass die Bindung von Hfq RNase E Schnittstellen und folglich auch die Prozessierung durch RNase E blockiert, aber keinen Einfluss auf die Bindungsfähigkeit von RsmA an RsmY hat. Dieses Ergebnis erklärt die Notwendigkeit von Hfq für die translationale Regulation durch RsmA.

2 SUMMARY

Small regulatory RNAs are an emerging group of regulators that modulate a wide range of physiological processes. In Bacteria they include mRNA leaders that affect expression in *cis*, small RNAs that bind to proteins or base-pair with target RNAs or DNA. sRNAs are also known to regulate virulence gene expression in several pathogenic bacteria, including *Pseudomonas aeruginosa*, *Vibrio cholerae* and *Staphylococcus aureus*. Due to genome wide detection methods the number of sRNAs is rising, but for the majority of them their function remains to be determined.

This work concentrated on the detection and characterization of novel sRNAs in *P. aeruginosa*. We used a shotgun-cloning approach (RNomics) as well as bioinformatic tools to identify novel sRNAs. The RNomics approach revealed three novel sRNAs and the *in silico* analyis, which was based on the evolutionary conservation of the RNA structure yielded seven potential sRNA candidates out of which two were shown to be expressed. An additional bioinformatic approach focussed on sRNA genes with an overall length between 80-300 nts preferably located in intergenic regions which are preceded by a promoter and followed by a Rho-independent terminator. This analysis yielded three candidate sRNAs, PaeI, PaeII and PaeIII.

The synthesis of the sRNA PaeII is governed by an RpoN-dependent promoter. PaeII is vigorously expressed under nutrient limiting conditions, and was shown to be dependent on guanosine tetraphosphate levels. A comparative analysis of the transcriptome profiles of the parental wt strain and the *paeII* deletion mutant revealed that the steady state levels of genes involved in purine biosynthesis were down-regulated in the presence of PaeII. We interpret this result as showing that PaeII serves to block purine biosynthesis during nutrient starvation.

PaeIII is preferentially expressed in stationary phase and upon challenge with non-inactivated human serum, conditions that are related to infection. A transcriptome analysis

upon *paeIII* over-expression revealed that expression of components of the MexX/Y multidrug efflux pump as well as that of the type III secretion apparatus is increased. In opposite, the expression of genes involved in pyocin and amino acid biosynthesis and genes involved in anaerobic respiration was decreased. Among the repressed genes was also *rpoS*, whose translation was shown to be regulated by PaeIII.

In addition, the effect of Hfq on the *P. aeruginosa* sRNAs RsmY and RsmZ was studied. Both sRNAs sequester the translational repressor protein RsmA, and thereby regulate the expression of virulence genes. Previous results have shown that Hfq binds to and stabilizes the sRNA RsmY, but not the functionally homologous sRNA RsmZ. Here, I could show that Hfq stabilizes RsmY by blocking RNase E cleavage and that binding of Hfq still permits RsmY- mediated titration of RsmA. These results explained why Hfq is required for full fledged translational regulation by RsmA.

3 INTRODUCTION

Small bacterial regulatory RNAs, also known as riboregulators, are regulatory elements that modulate gene expression at the post-transcriptional level (Waters and Storz, 2009). The majority of the identified sRNAs adjust bacterial physiology in response to environmental changes or stress conditions. One class of sRNAs base-pairs with mRNAs and changes their translation efficiency and/or stability. The second class of sRNAs interacts with proteins and modulates their activity. In addition, there is evidence that sRNAs binding to DNA can cause transcriptional attenuation (Giangrossi *et al.*, 2010).

sRNAs are either generated through processing or synthesized as primary transcripts. The size of "small" regulatory RNAs ranges from 50-500 nts. sRNAs are generally non-coding, but there are also known sRNAs that encode polypeptides and simultanously act as riboregulators. For instance, the *Staphylococcus aureus* RNAIII and *Escherichia coli* SgrS encode for the δ-hemolysin and SgrT protein, respectively (Janzon *et al.*, 1989; Wadler and Vanderpool, 2007). In pathogenic bacteria, such as *Pseudomonas aeruginosa*, sRNAs have been found to be involved in regulation of virulence gene expression. In addition to binding to proteins and mRNA, sRNAs can target DNA, as exemplified for the clustered regularly interspaced short palindromic repeats (CRISPR) in *Streptococcus thermophilus* (van der Oost *et al.*, 2009) and the *Shigella flexneri* sRNA RnaG (Giangrossi *et al.*, 2010).

3.1 Base-pairing sRNAs

3.1.1 *Cis-acting sRNAs*

The majority of *cis*-acting sRNAs which are transcribed convergently to their target genes are encoded by accessory genetic elements; plasmids, transposons and phages. Only a few have been found in the bacterial chromosome (Brantl, 2007). These RNAs are usually small (50-300 nt) and regulate biological processes such as replication, conjugation, suicide, transposition, mRNA degradation and translation initiation. In addition, some control

metabolic processes, such as the p3 RNA of *Clostridium acetobutylicum* that is involved in glutamine synthesis (Fierro-Monti *et al.*, 1992). Another sRNA class acts as antitoxin to repress translation of genes encoding toxic proteins (e. g. RatA/TxpA of *B. subtilis* (Silvaggi *et al.*, 2005)). The mechanisms of action (Fig. 1) include transcription attenuation, translation inhibition, inhibition of primer maturation, promotion or inhibition of mRNA degradation and inhibition of RNA pseudoknot formation (Brantl, 2007). A novel mechanism entails premature termination of transcription of the *cis*-encoded mRNA target *icsA* by RnaG in *Shigella flexneri* (Giangrossi *et al.*, 2010). *Cis*-encoded sRNAs are generally complementary to their target mRNAs. Therefore, the RNA-RNA interaction is usually strong and does not require proteins that facilitate annealing, such as the RNA chaperone Hfq. Tiling array approaches (Toledo-Arana *et al.*, 2009) as well as genomic SELEX (Lorenz *et al.*, 2006; Lorenz *et al.*, 2010) suggested that the number of chromosomally encoded *cis*-antisense RNAs is increasing. Long (> 1000 nt) RNAs spanning more than one gene as well as *cis*-acting sRNAs that overlap with the 5' or 3' UTR of mRNAs have been detected (Toledo-Arana *et al.*, 2009).

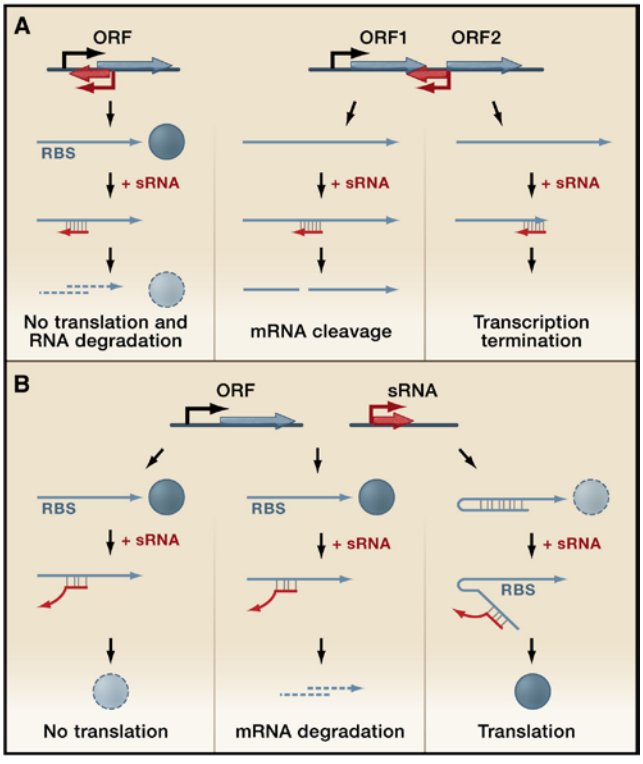


Fig. 1. Gene arrangement and regulatory functions of base pairing regulatory RNAs. (A) Regulatory mechanisms of *cis*-encoded antisense sRNAs (red) imposed on their target RNAs (blue). Base pairing of a sRNA encoded opposite to the 5' UTR inhibits ribosome binding and often leads to target mRNA degradation (left panel). Base-pairing of an sRNA encoded opposite to the sequence separating two genes in an operon to its target, can target RNases to this region and can cause mRNA cleavage or transcriptional termination (right panels) (B) Regulatory Functions of *trans*-encoded sRNAs. *Trans*-encoded sRNA can act negatively by base pairing with the 5' UTR and block ribosome binding (left panel) and/or targeting the sRNA-mRNA duplex for degradation by RNases (middle panel). *Trans*-encoded sRNA can act positively by preventing the formation of an inhibitory structure, which sequesters the ribosome-binding site (RBS) (right panel) (Waters and Storz, 2009).

3.1.2 *Trans*-acting sRNAs

In contrast to the aforementioned *cis*-encoded antisense RNAs, *trans*-acting sRNAs are generally encoded distantly from their target mRNA(s). The majority of the hitherto characterized sRNAs act as negative regulators by preventing ribosome loading onto the mRNA through base-pairing with, or in the vicinity of the ribosome binding site (Fig. 1). As a result, the respective mRNA is prone to rapid decay (for review, Gottesman, 2004; Kaberdin and Bläsi, 2006; Papenfort and Vogel, 2009). Variations of negative regulation by sRNAs exerted distantly from the Shine and Dalgarno (SD) sequence can further include binding to and obstructing of an upstream ribosome loading site (Darfeuille *et al.*, 2007), obstruction of a C/A rich element (Sharma *et al.*, 2007) as well as binding to the immediate (Bouvier *et al.*, 2008) or within the mRNA coding region (Pfeiffer *et al.*, 2009). Positive regulation by sRNAs appears to be less frequent. In this case the sRNAs act by an "anti-antisense" mechanism and compete with the formation of or melt intramolecular secondary structures, which block ribosome binding (Fig. 1, Morfeldt *et al.*, 1995; Repoila *et al.*, 2003; Urban and Vogel, 2008). In addition, indirect translation silencing by repressing translation of an upstream reading frame to which the target mRNA is positively translationally coupled has also been reported (Vecerek *et al.*, 2007). In the case of *V. cholerae* Qrr sRNA the same region is even used to activate and repress mRNAs (Lenz *et al.*, 2004).

Another hallmark of *trans*-acting sRNAs is their imperfect complementary with their mRNA targets. As a result, many sRNAs can interact with several mRNA targets (Papenfort and Vogel, 2009). This limited complementarity apears to be the cause for the requirement of

a dedicated RNA chaperone, which facilitates sRNA-mRNA interaction. *Trans*-encoded sRNAs are generally synthesized under certain growth or stress conditions (Repoila and Darfeuille, 2009). For instance the *E. coli* specific sRNAs are synthesized under low iron, oxidative stress, outer membrane stress, elevated glycine, changes in glucose concentration, and elevated glucose-phophate levels (Urbanowski *et al.*, 2000; Gottesman, 2005; Johansen *et al.*, 2006; Görke and Vogel, 2008; De Lay and Gottesman, 2009).

3.1.3 Hfq

The post-transcritional regulator Hfq was first discovered as a host factor required for replication of the bacteriophage Qβ (Franze de Fernandez *et al.*, 1968). It belongs to the eukaryotic and archaeal family of Sm- and Sm-like proteins (Møller *et al.*, 2002; Sun *et al.*, 2002; Zhang *et al.*, 2002). The size of thermostable Hfq proteins ranges from 70 to 110 amino acids in different bacterial species and forms homo-hexamers (Fig. 2, Møller *et al.*, 2002; Schumacher *et al.*, 2002; Zhang *et al.*, 2002; Sauter *et al.*, 2003; Valentin-Hansen *et al.*, 2004; Nikulin *et al.*, 2005; Link *et al.*, 2009). Recent studies suggested, that the sRNA binds to the proximal side of the hexamer (Fig. 2, Schumacher *et al.*, 2002; Mikulecky *et al.*, 2004), whereas the C-terminal extension constitutes a mRNA interaction surface (Vecerek *et al.*, 2008). These observations support a model in which the sRNA and the mRNA bind concomitantly to Hfq, which in turn results in an increased local concentration, aiding the RNA-RNA interaction. Furthermore, this would explain why bacterial Hfq proteins with short C-termini seem not to contribute to sRNA- mRNA interactions. Unlike uridine-containing sequences poly(A) tract binds to the distal face of Hfq (Link *et al.*, 2009).

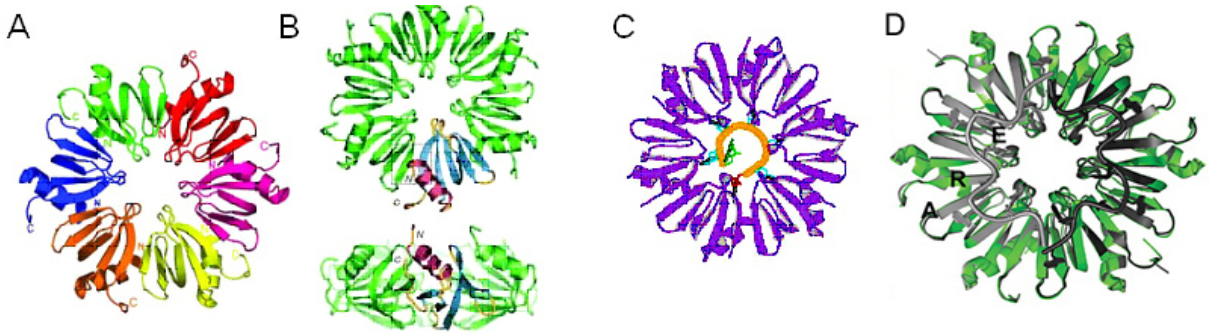


Figure 2. (A) The structure of the Hfq protein from *P. aeruginosa*. Top view of the Hfq hexameric ring (Nikulin *et al.*, 2005). (B) Structure of the N-terminal 72 aa of *E. coli* Hfq. Top and side views of the Hfq hexameric doughnut. Secondary structure elements are highlighted in one monomer with the N-terminal α-helix in pink and the five ß-strands in blue (Sauter *et al.*, 2003). (C) Structure of *S. aureus* Hfq bound to a hepta- oligoribonucleotide (orange) (Schumacher *et al.*, 2002). (D) A ribbon diagram of the Hfq hexamer and A_{18} oligonucleotide superimposed onto the apo Hfq hexamer (green). The Hfq trimers and A_9 oligonucleotides of the 2-fold related asymmetric units are gray and black, respectively. One of the 6 tripartite binding repeats is labeled A (adenosine site), R (purine nucleotide site), and E (entrance/exit site) (Link *et al.*, 2009).

This protein preferentially binds to AU-rich single stranded regions either preceded or followed by a stem-loop structure (Brescia *et al.*, 2003; Moll *et al.*, 2003b; Mikulecky *et al.*, 2004; Sun and Wartell, 2006; Lorenz *et al.*, 2010). Hfq is one of the most abundant proteins in *E. coli* with 30,000- 60,000 copies per cell (Kajitani *et al.*, 1994). The high number of molecules may reflect the pleitropic effects of *hfq* mutants, including decreased growth rates, increased oxidation of carbon sources, increased sensitivity to ultraviolet light, mutagens and antioxidants as well as abbarant cell growth (Tsui *et al.*, 1994). The level of Hfq was reported to decrease gradually during transition from logarithmic to stationary phase (Kajitani *et al.*, 1994; Azam and Ishihama, 1999). Contrary to these findings, the cellular amount of Hfq has also been reported to increase under slow growth conditions and upon entry in stationary phase (Tsui *et al.*, 1997; Vytvytska *et al.*, 1998; Sonnleitner *et al.*, 2006). The expression of Hfq is controlled at the transcriptional and at the translational level. The *hfq* gene is part of a super-operon that encompasses at least three σ^{32} "heat shock" promoters in addition to four σ^{70}-dependent promoters. They ensure that high Hfq levels are maintained during stress conditions. It also seems to modulate its own mRNA stability, and acts as an autogeneous translational repressor (Tsui *et al.*, 1997; Vecerek *et al.*, 2005).

In addition to sRNA-dependent regulation, Hfq modulates the decay of some mRNAs by binding to their poly(A) tails. Hfq has been shown to stimulate poly(A) adenylation by poly(A) polymerase I, and to enhance degradation of the RNA (Hajnsdorf and Regnier, 2000). On the other hand Hfq also protects messages from polynucleotide phosphorylase, RNase II and RNase E (Folichon *et al.*, 2003; Mohanty *et al.*, 2004; Folichon *et al.*, 2005). Hfq was also reported to have an ATPase activity, which could be linked to its RNA chaperone activity (Sukhodolets and Garges, 2003).

Hfq was shown to protect the sRNAs RyhB and DsrA RNAs from cleavage by RNase E (Massé *et al.*, 2003; Moll *et al.*, 2003a), the major endonuclease involved in mRNA decay as well as in the processing of tRNA and rRNA precursors. This observation can probably be extended to many riboregulators, because Hfq binding sites appear to coincide with recognition sites for RNase E, both of which recognize AU-rich sequences (Massé *et al.*, 2003; Moll *et al.*, 2003a; Sorger-Domenigg *et al.*, 2007).

Phylogenetic analyses revealed Hfq orthologues in many Gram-positive (*Chlamydia muridarum, Chlamydia trachomatis, Chlamydophlia pneumoniae, Borrelia burgdorferi, Treponema pallidum, Cytophaga hutchinsonii, Deinococcus radiodurans, Mycobacterium leprae, Thermobifida fusca, Streptomyces coelicolor, Nostoc sp., Nostoc punctiforme Synechocystis sp., Prochlorococcus marinus, Synechococcus sp, Bacillus halodurans, Mycoplasma genitalium, Bacillus subtilis, Clostridium acetobutylicum, Clostridium perfringens, Ureaplasma urealyticum, Listeria innocua, Lactococcus lactis, Listeria monocytogenes, Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus pyogenes, Bacillus anthracis, Enterococcus faecium*) and Gram-negative Bacteria (α/β/γ/ε Proteobacteria, *Prochlorococcus marinus, Synechocistis* PCC6803) (Sun *et al.*, 2002) as well as in Archaea (*Methanococcus jannaschii* (Nielsen *et al.*, 2007); *Sulfolobus solfataricus* (Kilic *et al.*, 2005)). The function of Hfq in Gram-positive bacteria is unknown, because except for *L. monocytogenes* (Nielsen *et al.*, 2009), it is either absent (*Streptococcus pyogenes, Borrelia*

burgdorferri, Enterococcus faecalis, Streptomyces coelicolor, Chlamydia trachomatis) or seems to be dispensable (*Bacillus subtilis, Staphylococcus aureus*) for sRNA controlled gene regulation (Jousselin *et al.*, 2009). So far, no additional RNA chaperones have been discovered, which might substitute for the function of Hfq in Gram-positive bacteria. It has been proposed that a link exists between the need for Hfq in sRNA-mRNA interactions and (i) the overall GC content of bacterial genomes, (ii) the free energy for sRNA-mRNA pairing interactions, (iii) genome size and (iv) structural variations among Hfq proteins (Jousselin *et al.*, 2009).

Hfq has been identified as a virulence factor in *Yersinia enterocolitica, Brucella abortus, Legionella pneumophila, Salmonella typhimurium* and *Vibrio cholerae* (Nakao *et al.*, 1995; Robertson and Roop, 1999; Ding *et al.*, 2004; McNealy *et al.*, 2005; Sittka *et al.*, 2007). A Pseudomonas *hfq* mutant showed a reduced virulence in larvae of *Galleria mellonella* and in mice (Sonnleitner *et al.*, 2003). As in *E. coli* (Sledjeski *et al.*, 2001), Hfq is involved in regulation of *rpoS* expression in PAO1 (Sonnleitner *et al.*, 2003). However, Hfq also exerts σ^S-independent effects on virulence factors, such as catalase, pyocyanin and elastase production, and is required for PAO1 to swarm and twitch, which are important traits for colonization and biofilm development (Sonnleitner *et al.*, 2003). A comparative transcriptome analysis of a PAO1 *rpoS*– and a *rpoS–hfq*– strain has indicated that Hfq affects approximately 5 % of the PAO1 transcriptome (Sonnleitner *et al.*, 2006). Among these transcripts, 72 are known to be regulated by quorum sensing (QS), which is a cell density-dependent regulatory mechanism that impacts on virulence gene expression (van Delden and Iglewski, 1998).

3.1.4 Regulatory RNAs that modulate protein activity

In contrast to protein-binding sRNAs with intrinsic catalytic activity (like RNase P (Kazantsev and Pace, 2006)), CsrB/C (Babitzke and Romeo, 2007), 6S (Wassarman, 2007) and GlmY (Görke and Vogel, 2008) act by antagonizing the function of their cognate proteins through mimicking the structures of other nucleic acids (Fig. 3).

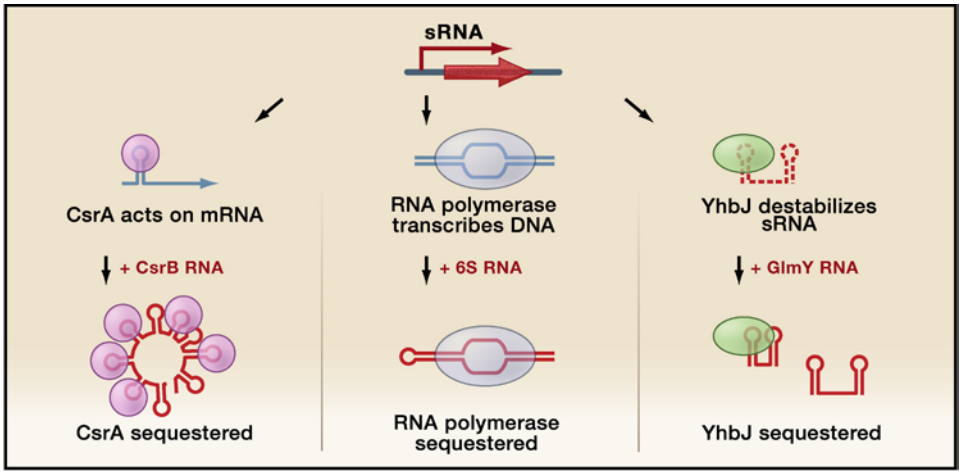


Fig. 3. Protein-binding sRNAs (red) that antagonize regulatory proteins (Waters and Storz, 2009).

6S RNA accumulates during exponential growth and reaches elevated levels when cells enter stationary phase. It sequesters the σ^{70}-containing RNA-polymerase, and thus provokes a reprogramming of the transcriptional activity. At outgrowth of stationary phase the NTP levels increase to a threshold, and subsequently 6S is used as a template for the transcription of pRNA, which leads to dissociation of the 6S-pRNA complex. As a consequence, all σ^{70}-dependent promoters can be re-used (Wassarman, 2007). 6S is widespread among bacteria, with *B. subtilis* carrying even two 6S RNA genes that interact with the houskeeping form of RNA-polymerase and a third one with a distinct RNA-polymerase binding activity (Trotochaud and Wassarman, 2005). A phylogenetic comparison revealed a highly conserved secondary structure without notable conservation of the primary sequence (Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005).

The *E. coli* CsrB/C sRNAs sequester the translational repressor CsrA, the central regulator of carbon storage. The CsrA dimer binds to GGA-motifs in the 5'-UTR of target mRNAs and thereby blocks translation. The BarA-UvrB two-component system senses an hitherto unknown signal under poor growth conditions, which is possibly pH-dependent (Mondragon *et al.*, 2006), and thereby induces the expression of the sRNAs CsrB/C (Gudapaty *et al.*, 2001; Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003). The sRNAs carry

multiple GGA motifs, which are necessary to sequester CsrA, and thus prevents binding of CsrA to translation initiation regions. In *E. coli*, the CsrB/C stability is regulated through CsrD that recruits RNase E to degrade the sRNAs (Suzuki *et al.*, 2006). Regulatory circuits homologous to the CsrA/B/C system are found in a broad range of γ-proteobacteria including *Salmonella*, *Erwinia*, *Vibrio*, *Yersinia*, *Legionella*, *Haemophilus* and *Pseudomonas*, where they impact on secondary metabolism, quorum sensing and epithelial cell invasion (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008; Lucchetti-Miganeh *et al.*, 2008).

The sRNA GlmY was recently proposed to compete with YhbJ, a factor that binds to a highly similar sRNA GlmZ to induce its cleavage (Görke and Vogel, 2008). These two seemingly homologous sRNAs act hierarchically to activate *glmS* translation, which encodes glucosamine-6-phosphate synthetase. The *glmS* mRNA is derived from the bicistronic mRNA *glmUS* after processing by RNase E. The proposed model specifies that an inhibitory stem-loop structure in the 5'-UTR of *glmS* is relieved upon binding of GlmZ, and thereby frees the SD-sequence and makes it accessible for ribosome-binding. The highly homologous sRNA GlmY lacks the *glmS* complementary region, and therefore indirectly activates *glmS* expression through inhibition of GlmZ processing, which renders GlmZ unable to activate *glmS* translation (Kalamorz *et al.*, 2007; Reichenbach *et al.*, 2008; Urban and Vogel, 2008).

3.1.5 CRISPR-Clustered regularly interspaced short palindromic repeats

Clustered regularly interspaced short palindromic repeats (CRISPRs) are a novel class of repetitive DNA elements, where the repeats are interspersed with spacers homologous to DNA from phages and plasmids (Sorek *et al.*, 2008). Adjacent to those clusters CRISPR-associated (Cas) genes are encoded, which are needed for CRISPR activity. It has been shown that integration of a prophage into the CRISPR array confers resistance to subsequent phage infections (Barrangou *et al.*, 2007). This interspaced DNA is transcribed into a long RNA, which is processed to by the *cas* gene products into single spacers in a manner reminiscent to

the eukaryotic RNAi pathway. They directly base-pair with cognate nucleic acid targets with DNA rather than RNA being the candidate for target interference (Waters and Storz, 2009).

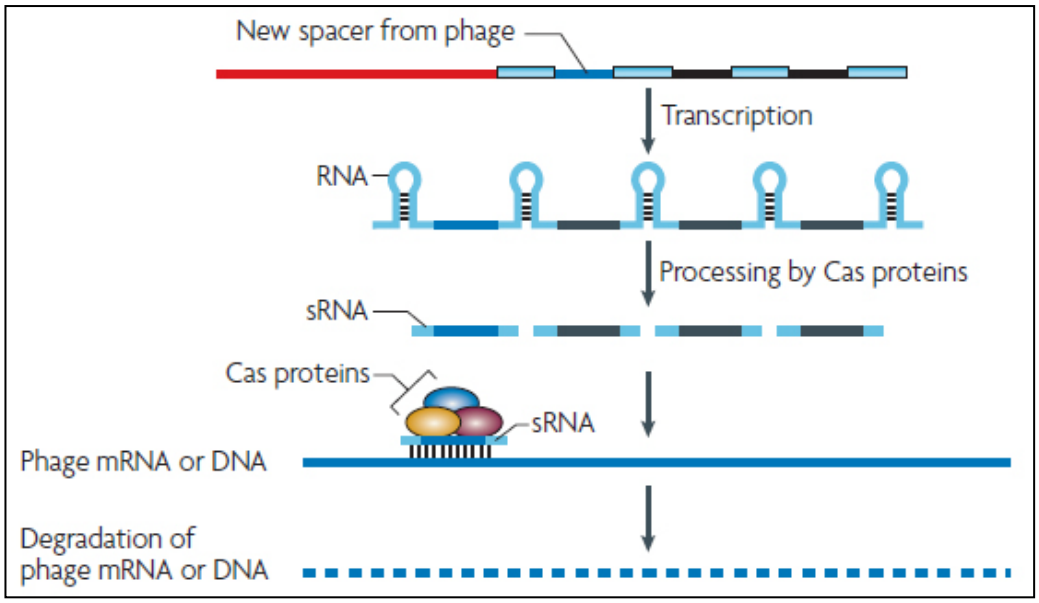


Fig. 4.. Putative, simplified model for CRISPR action (Sorek *et al.*, 2008). See text.

Although many details remain poorly understood, it has become clear that the CRISPR–Cas system is a specific, dynamic and inheritable protection system in prokaryotes that provides them with immunity towards accessory genetic elements (van der Oost *et al.*, 2009).

3.2 How to find regulatory RNAs

The first sRNAs were identified incidentally through the use of genetic screens or through radiolabelling of total RNA and subsequent isolation from gels (Wassarman *et al.*, 1999). Especially, highly expressed and housekeeping sRNAs such as the 4.5S RNA component of the signal recognition particle, the 6S RNA, which modulates RNA polymerase activity, M1 RNA, the catalytic subunit of RNase P, tmRNA that releases stalled ribosomes on defective mRNAs and the antisense regulator Spot42 have been discovered in this way. The majority of sRNAs has been discovered in the last decade by systematic genome wide screens that were based on: (i) computational prediction (ii) direct detection using microarrays (iii) direct isolation, including shotgun cloning (RNomics) and (iv) co-purification with proteins (Vogel and Sharma, 2005; Altuvia, 2007).

The identification of sRNAs by biocomputation is challenging, due to a lack of conserved features among sRNAs. In many bacterial species the consensus sequences of several sigma factors and transcriptional regulators are ill defined, and are therefore not well suited as search parameters. Most multilayered bioinformatic approaches concentrate on intergenic regions and the conservation of the primary sequence or the secondary structure, which is preceded by a promoter and followed by a Rho-independent terminator. *Cis*-encoded and sRNAs that overlap with coding regions are overlooked by these bioinformatical approaches. Most search algorithms use parameters that were deduced from the first identified sRNAs in *E. coli* and are validated by counting the number of previously known sRNAs that they were able to detect. However, each of them fails to identify all the experimentally verified *E. coli* sRNAs (Pichon and Felden, 2008).

Genome-wide detection of sRNAs by experimental strategies was facilitated by the increased availability of tiling arrays and the fast progress in high-throughput cDNA sequencing (Sharma and Vogel, 2009). High-density oligonucleotide arrays that cover sense and anti-sense strand including the intergenic regions allow a comparison of the transcriptome under various growth conditions and from different mutant strains. This method revealed differential expression of sRNAs in the presence and absence of Hfq in a cyanobacterial strain (Steglich *et al.*, 2008), as well as sRNAs of virulent bacteria that are up-regulated during and required for successful host infection (Akama *et al.*, 2009; Toledo-Arana *et al.*, 2009). sRNAs can be pre-selected by virtue of binding to Hfq (Tjaden *et al.*, 2002; Zhang *et al.*, 2003; Sonnleitner *et al.*, 2008). To reduce the number of rRNAs in the RNomics pool, the ribosomes can be removed by ultracentrifugation (Sonnleitner *et al.*, 2008), or the clones can be pre-selected with rRNA or tRNA specific probes prior to sequencing (Vogel *et al.*, 2003). Depletion for rRNA can also be achieved by employing special RNA extraction kits (Ambion MICROBExpress). Recently, a protocol for the elimination of small-sized 5S rRNA and tRNAs by oligonucleotide-guided RNAse H treatment was introduced (Liu *et al.*, 2009).

DNA microarrays and shotgun cloning share the same drawback because they require cDNA synthesis, which selects for highly abundant RNAs and for less structured and/or modified sRNAs that are more easily transcribed by reverse transcriptase. A novel detection method employing antibodies specific for RNA-DNA hybrids circumvents the problem of DNA microarrays (Hu *et al.*, 2006). An advantage of high-throughput sequencing and tiling arrays is that bacterial transcriptomes can be profiled in a quantitative manner (Passalacqua *et al.*, 2009).

It is known from many sRNAs that they stably associate with cellular proteins and can therefore be co-purified. Examples include *E. coli* CsrB and *P. fluorescens* RsmZ which co-purified with their target proteins CsrA and RsmA, respectively (Liu *et al.*, 1997; Heeb *et al.*, 2002). Total RNA pools which are enriched for sRNAs can be analyzed by hybridization to tiling arrays (Zhang *et al.*, 2003), by direct sequencing (Christiansen *et al.*, 2006) or by conventional RNomics (Sonnleitner *et al.*, 2008).

In the genomic SELEX approach developed in the Schroeder laboratory (Lorenz *et al.*, 2006) a library of random sequences idealy covering the whole *E. coli* genome is transcribed by T7 Polymerase, and then the RNA is selected for Hfq binding. The retained RNA was then converted to cDNA and subjected to additional rounds of selection and amplification. This approach has the advantage that RNAs are generated independently of growth conditions and that low abundant antisense RNAs can be detected. However, only a few known Hfq-associated sRNAs were identified (Lorenz *et al.*, 2006), which raises the question of the biological significance of this approach.

3.3 sRNA target search

Because of the emergence of high-throughput screens the number of identified sRNAs is rapidly increasing. The remaining challenge is to identify their cellular interaction partners or target mRNAs. Unlike *cis*-encoded anti-sense RNAs (Brantl, 2007), *trans*-encoded anti-sense

RNAs typically have only limited complementarity to their mRNA targets. Activation sites are usually more than 20 nucleotides upstream of the translational start site (Fröhlich and Vogel, 2009), whereas repression commonly takes generally place in close proximity to the start codon (Waters and Storz, 2009). As mentioned above sRNAs can also act in the coding region (Bouvier *et al.*, 2008; Papenfort *et al.*, 2009; Pfeiffer *et al.*, 2009) or even in the 5' UTR (Darfeuille *et al.*, 2007). Therefore, it is difficult to predict the outcome of regulation by the location of the interaction site. Furthermore, sRNAs do not exclusively repress or activate translation, but can perform either function on different targets. For instance the *E. coli* sRNA DsrA can repress *hns* mRNA (Lease *et al.*, 1998) and activate *rpoS* mRNA (Lease *et al.*, 1998; Sledjeski *et al.*, 2001; Resch *et al.*, 2008).

Biocomputational approaches for sRNA target search make use of considerations that can be incorporated into algorithms such as the lenght and position of the base-paired sequence, structural and sequence conservation as well as the location of the target site. Web-based platforms like TargetRNA (Tjaden *et al.*, 2006) calculate the optimal hybridization score and then rate the results. However, the paucity to detect known sRNA-mRNA interactions limits several of these approaches. In addition, bioinformatical methods can not predict protein interaction partners.

The comparison of transcriptome profiles of strains upon differential expression of the sRNA can be used to find mRNA targets. It relies on the fact that mRNAs can be stabilized by translation or subject to coupled degradation of the sRNA and its mRNA target. To increase the chance of detecting direct sRNA targets over indirect secondary targets, sRNA expression can be induced from a controllable promoter for a short time interval (10- 20 min) followed by microarray analysis (Vogel and Wagner, 2007).

Proteomic approaches comparing strains lacking or over-expressing a sRNA resulted in the identification of the MicA target *ompA* (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). An

inherent problem of this method is the low number of resolved proteins on 2D gels and that it is not possible to distinguish direct from indirect targets.

Direct target mRNA capturing using the sRNA as bait was successfully used in two studies. One used Hfq-sRNA complexes which were incubated with a pool of extracted mRNAs and the retained fragments were converted to cDNA and analysed (Antal *et al.*, 2005). In another approach the sRNA was bound to streptavidin magnetic beads, which were incubated with extracted mRNAs, and the bound fragments were converted to cDNA and hybridized to whole genome microarrays (Douchin *et al.*, 2006).

To identify protein targets fishing experiments can be employed using the sRNA as bait. This was done in the case of the plasmid ColE1 encoded sRNA Rcd, which was affinity tagged and bound to the *E. coli* protein tryptophanase (Chant and Summers, 2007). It appears to increase the affinity of the enzyme for its substrate tryptophan and thereby stimulates indole synthesis (Chant and Summers, 2007).

Genetic approaches involving transposon mutagenesis rarely hit sRNA genes, because of their small size and because most sRNAs are not essential under standard growth conditions. For target discovery reporter gene fusions in combination with plasmid libraries carrying cloned sRNA genes or genomic fragments can be used for the identification of sRNA targets. This approach was successful for RybC, which targets the genes encoding the two component system DpiA/B (Mandin and Gottesman, 2009).

Regardless of the route taken for target identification, the targets have to be validated. Site specific mutagenesis at the interaction site of the sRNA or the target should abolish regulation, which should be re-established by compensatory mutations. With the help of target reporter gene fusions (*lacZ, gfp*) the effect of a sRNA on the target mRNA translation can be assessed. If the target fusion is driven by the authentic promoter effects on transcriptional level have to be ruled out by independent experiments. Urban and Vogel (2009) constructed a set of vectors to confirm target mRNAs, which can be used to study sRNA-target interaction

of different bacteria in *E. coli*. *In vitro* approaches (*in vitro* translation, bandshifts, toeprinting, footprinting) can be applied to verify the *in vivo* interaction studies.

3.4 *Pseudomonas aeruginosa* regulatory RNAs

3.4.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (family Pseudomonaceae) is a Gram-negative, rod shaped, aerobic, motile bacterium, which is known for its ability to survive in many habitats; in water, soil, and vegetation. Being an opportunistic human pathogen, *P. aeruginosa* is the most common Gram-negative bacterium found in nosocomial infections. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in immunosuppressed patients. Patients hospitalized with cancer, AIDS, cystic fibrosis and severe burns are particularly prone to *P. aeruginosa* infections (van Delden and Iglewski, 1998).

The capacity of *P. aeruginosa* to produce such diverse infections is due to an arsenal of virulence factors (Fig. 5) (van Delden and Iglewski, 1998). *P. aeruginosa* produces cell-associated (flagellum, pilus, nonpilus adhesins, alginate, lipopolysaccharide) and extracellular virulence factors (proteases, hemolysins, exotoxin A, exoenzyme S, hydrogen cyanide and pyocyanin).

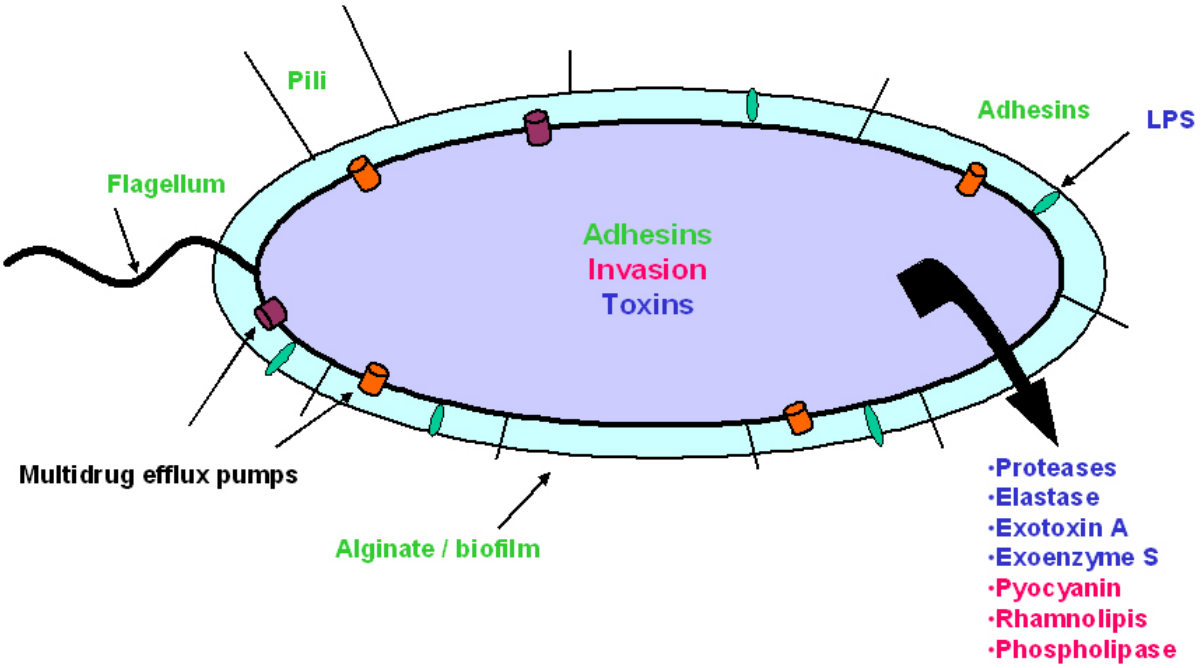


Fig. 5. Virulence factors of *P. aeruginosa* (modified from van Delden and Iglewski, 1998).

To initiate the infection, *P. aeruginosa* requires a substantial loss of first-line defenses by the host. Adherence to epithelium is probably mediated by type IV pili and several other non-pilus adhesins. In addition, flagella, which are primarily responsible for motility, may act as adhesins to epithelial cells (Feldman *et al.*, 1998). *P. aeruginosa* produces several extracellular products that, after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination. These include (i) exotoxin A (Wick *et al.*, 1990), which is responsible for local tissue damage, bacterial invasion, and possibly immunosuppression, (ii) two hemolysins, phospholipase C and rhamnolipid, which may act synergistically to break down lipids and lecithin, and thereby contribute to tissue invasion due to their cytotoxic effects, and (iii) several proteases including LasB elastase, LasA elastase, and alkaline protease (Gambello *et al.*, 1993; Toder *et al.*, 1994). *P. aeruginosa* uses secretion systems to transport virulence factors. The type III secretion system is a powerful tool to directly inject effector proteins (ExoS, ExoT, ExoU and ExoY) into the host cell (Hauser, 2009).

P. aeruginosa shows resistance against many structurally unrelated antibiotics. Resistance mechanisms include low outer membrane permeability, multidrug efflux pumps (tetracycline,

imipenem, flouroquinolones, aminoglycosides) and the production of antibiotic modifying enzymes (aminoglycosidases, β-lactamases) (Nakae, 1995; Nikaido, 1996; Piddock, 2006). In addition, the production of alginate and the ability of *P. aeruginosa* to form biofilms protects the bacteria from therapeutic intervention (Davies *et al.*, 1998).

P. aeruginosa is a highly adaptable organism with a large dynamic genome (Mathee *et al.*, 2008), approximately 10% of which is devoted to regulatory elements that tightly regulate the production of virulence factors, secondary metabolites, expression of multidrug efflux pumps and biofilm maturation. Those regulatory elements comprise quorum sensing, the GacS-GacA two-component system, alternative sigma factors (RpoS, RpoN) and the post-transcriptional repressor protein RsmA (Juhas *et al.*, 2005). *P. aeruginosa* developed a complex quorum sensing cascade to communicate, to sense environmental changes and to modulate inflammatory and immune responses in mammals (Cooley *et al.*, 2008). It consists of three interlinked systems (Las, Rhl, PQS) employing acyl-homoserinelactones and alkyl quinolones as signalling molecules (Williams and Camara, 2009). The quorum sensing systems are interlinked and modulated by a large number of regulators, such as RpoS, RsmA, MvaT, PtxR, GacA, RpoN and Hfq.

3.4.2 *P. aeruginosa* housekeeping sRNAs

P. aeruginosa shares a number of "housekeeping sRNAs" with *E. coli*, including 6S RNA (Vogel *et al.*, 1987; Sonnleitner *et al.*, 2008), which is needed for transcriptional reprogramming in the transition from exponential to stationary phase of growth and *vice versa* (Wassarman, 2007). It was experimentally verified in *P. aeruginosa* (Vogel *et al.*, 1987) and appeared in a bioinformatic screen, which did not rely on the presence of Rho-independent terminators (Sonnleitner *et al.*, 2008). The primary and secondary structure of *P. aeruginosa* 6S RNA is similar to the one of *E. coli* (Vogel *et al.*, 1987), and therefore similar mechansm can be hypothesized.

4.5S RNA is part of the signal recognition particle, which is needed to target proteins to the cytoplasmatic membrane. *P. aeruginosa* 4.5S RNA was identified in 2 bioinformatic screens (Gonzalez *et al.*, 2008; Sonnleitner *et al.*, 2008) but has not been experimentally verified. *P. aeruginosa* 4.5S RNA has 75% sequence identity with *E. coli* 4.5S RNA, and thus likely has the same function in both organisms. *E. coli* 4.5S RNA is processed from a longer precurser by RNase P, an enzyme that contains a catalytic RNA (*rnpB*). Pseudomonal *rnpB* has been first isolated from *P. fluorescens* (James *et al.*, 1988) but was also detected by three bioinformatical screens (Livny *et al.*, 2006; Gonzalez *et al.*, 2008; Sonnleitner *et al.*, 2008) in *P. aeruginosa*.

Transfer-messenger RNA (tmRNA) has features of tRNA and mRNA (Keiler, 2007). In case of ribosome stalling, it is charged with alanine and enters the ribosomal A-site. The nascent polypeptide is then transferred to the alanyl-tmRNA and a small ORF encoded by the mRNA part of tmRNA, is subjected to further eleongation and tags the polypeptide for proteolysis. In case of premature termination or mRNA truncation this mechanism ensures the release of stalled ribosomes and degradation of the truncated and tagged polypeptide. The tag sequence of *P. aeruginosa* tmRNA is similar to the one of *E. coli*, which made it possible to clone *P. aeruginosa* tmRNA (Williams and Bartel, 1996).

3.4.3 Protein sequestering sRNAs

3.4.3.1 RsmX/Y/Z

The sRNAs of the Rsm system RsmX/Y/Z (homologues of *E. coli* CsrB/C) modulate the activity of the translational repressor protein RsmA (Lapouge *et al.*, 2008). In pseudomonads the homodimers RsmA and RsmE (*P. fluorescens*) negatively control expression of virulence factors by binding to GGA motifs in the leader of their target mRNAs (Lapouge *et al.*, 2007; Schubert *et al.*, 2007). In addition, the stationary and stress sigma factor *rpoS* is either directly or indirectly under control of RsmA, which greatly expands the number of genes and processes governed by the Rsm system (Heeb *et al.*, 2005).

Expression of the sRNAs RsmX/Y/Z is induced by the GacA/GacS two component system, which is modulated by the two hybrid sensor kinases RetS and LadS (Ventre *et al.*, 2006). RetS has been shown to be pivotal for the transcription of genes associated with cytotoxicity and acute infections including the type III secretion system (Goodman *et al.*, 2004; Laskowski *et al.*, 2004; Ventre *et al.*, 2006). On the other hand RetS negatively controls transcription of genes which are associated with the synthesis of biofilm oligosaccharides (Goodman *et al.*, 2004; Ventre *et al.*, 2006). It directly interacts with GacS (Goodman *et al.*, 2009) and acts as an antagonist of GacA/S by blocking RsmZ transcription (Ventre *et al.*, 2006). RetS seems to be temperature-sensitive in *P. fluorescens* (Humair *et al.*, 2009). The kinases LadS and GacS, on the other hand, directly counteract RetS, as they stimulate the expression of the *psl* and *pel* operons by upregulating the expression of the RsmA-antagonist RsmZ (Kay *et al.*, 2006; Ventre *et al.*, 2006). This signal transduction network in which the activities of the signal-receiving sensor kinases LadS, RetS, and GacS regulate expression of virulence at the transcriptional and posttranscriptional level provides a “switch” between acute and chronic infection phenotypes (Fig. 6., Ventre *et al.*, 2006).

Furthermore, the sRNAs can inhibit their own transcription through a feedback mechanism by interfering with the GacS/GacA system, most probably by allosterically inhibiting GacA phosphorylation or by binding to the putative GacA box (Lapouge *et al.*, 2008). It was shown that the A+T-rich DNA segment upstream of *rsmZ* is bound and silenced by MvaT and MvaU, the global gene regulators of the H-NS family (Brencic *et al.*, 2009). In addition, in *P. aeruginosa* the sigma factor RpoN affects GacA expression in a negative manner (Heurlier *et al.*, 2003).

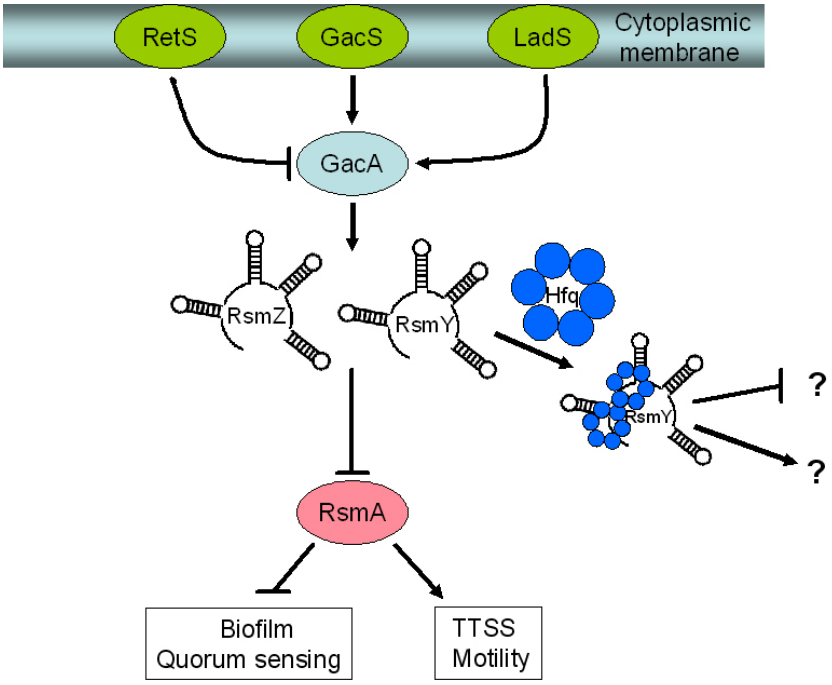


Fig. 6. General mechanism of the Rsm/Gac pathway in *P. aeruginosa*. ↓, positive effect; ⊥, negative effect; ?, unknown target. In response to environmental signals the sensor kinases GacS and LadS phosphorylate the response regulator GacA which leads to transcriptional activation of the sRNAs RsmY and RsmZ. The hybrid sensor kinase RetS acts in opposite by blocking autophosphorylation of GacS. The sRNAs are able to sequester the translational repressor RsmA, thereby preventing translational regulation of RsmA target mRNAs. RsmA activates type III secretion, pilus formation and mobility but represses biofilm formation and quorum sensing. RsmY interacst with and is stabilized by Hfq, which is known to facilitate base-pairing of sRNAs with their mRNA targets. Potential targets for RsmY besides sequestering RsmA are currently unknown (modified from Sonnleitner *et al.*, 2010).

The GacA regulated sRNAs vary in size between 100 and 479 nt, the largest known being RsmB of *Erwinia carotovora* (Babitzke and Romeo, 2007). All of them possess multiple GGA motifs that are located in single-stranded RNA regions, mostly in loops or between stem-loop structures. These motifs were shown to be required for interaction with RsmA (Valverde *et al.*, 2004). *P. aeruginosa* contains two RsmA binding sRNAs, RsmY and RsmZ (Kay *et al.*, 2006), whereas *P. fluorescens* contains three RsmX, RsmY and RsmZ (Kay *et al.*, 2005), all of which function as antagonists of RsmA. The synthesis of the sRNAs as well as of RsmA and/or RsmE inreases throughout cell growth, whereas *rsmZ* expression is observed later in the growth cycle (Kay *et al.*, 2005). It has been observed that RsmA and RsmE strongly stabilize the sRNAs, possibly through protection from nucleases (Reimmann *et al.*,

2005; Kay *et al.*, 2006). Moreover, it was shown that binding of Hfq stabilizes RsmY (Sonnleitner *et al.*, 2006) and that RsmA and Hfq can concomitantly bind to RsmY (Sorger-Domenigg *et al.*, 2007). Binding of Hfq to RsmY was shown to protect the sRNA from RNase E cleavage *in vitro* (Sorger-Domenigg *et al.*, 2007). This effect of Hfq on the sRNA could only be observed for *P. aeruginosa* RsmY but not for its homologue RsmZ (Sonnleitner *et al.*, 2006).

3.4.3.2 CrcZ

Catabolite repression prevents transcription of genes required for degradation of the less-preferred substrate in the presence of the preferred substrate (Ullmann, 1996; Görke and Stülke, 2008). In *P. aeruginosa* the RNA binding protein Crc is responsible for regulation of genes involved in sugar degradation pathways, e.g. *amiE*, encoding aliphatic amidase (Sonnleitner *et al.*, 2009). Crc acts as a translational repressor by binding to CA-motifs in the vicinity of the ribosomal binding site of target mRNAs. The similarity to RsmA in terms of translational repression led Sonnleitner *et al.* (2009) to the hypothesis that Crc could be antagonized by sRNAs. A screen in intergenic regions for CA motifs led to the discovery of the sRNA CrcZ which is transcribed in inverse orientation to the previously published P30 sRNA (Livny *et al.*, 2006) and contains five CA- rich motifs (Sonnleitner *et al.*, 2009). The sRNA CrcZ was shown to antagonize Crc in catabolite repression and is induced in a RpoN-dependent manner by the CbrA/B two component system in the presence of a non-preferred carbon source, such as mannitol (Sonnleitner *et al.*, 2009). CrcZ binds to Crc and thereby alleviates the negative control exerted by Crc on target mRNAs (Fig. 7).

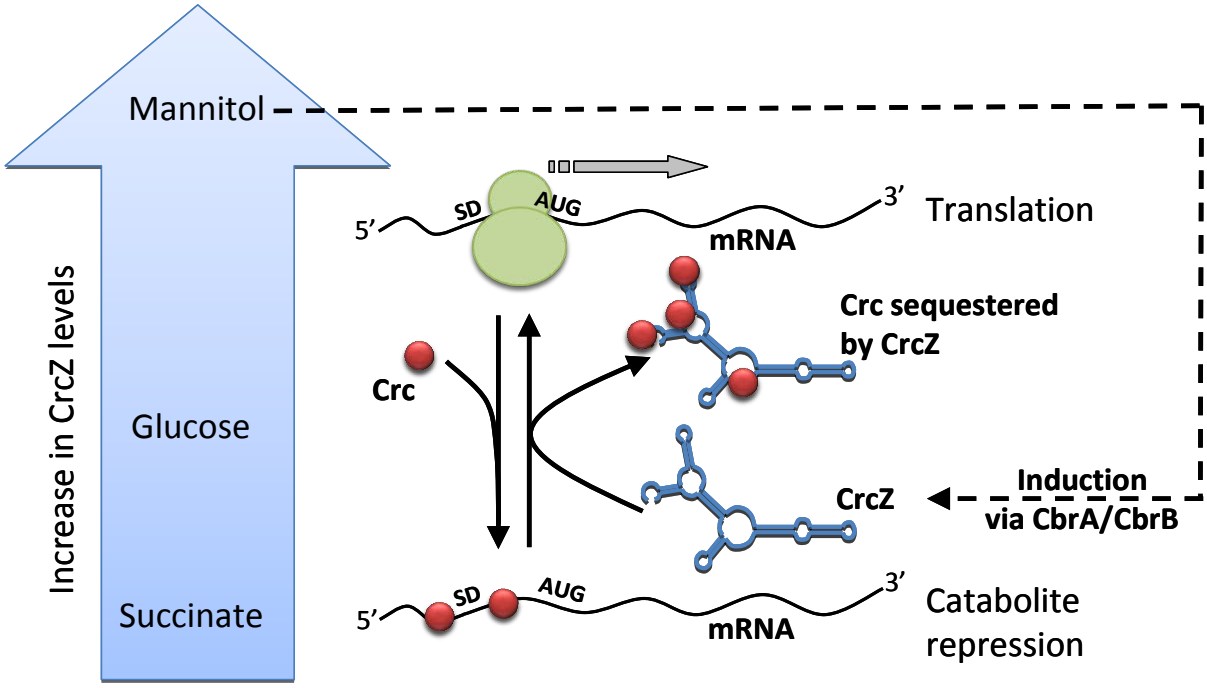


Fig. 7. Model of CrcZ as an antagonist of Crc in catabolite repression (Sonnleitner *et al.*, 2009). In the presence of the preferred carbon source Crc binds to catabolite-repression sensitive mRNAs (e. g. *amiE*) and blocks ribosome binding. In the presence of a non-preferred carbon source, such as mannitol, the CbrA/CbrB two component-system induces expression of the sRNA CrcZ, which sequesters Crc and thereby allows translation of target mRNAs.

Interestingly, CrcZ does not have a Rho-independent terminator stem-loop, and thus might depend on Rho (Sonnleitner *et al.*, 2009).

3.4.4 *P. aeruginosa* sRNAs

In pseudomonads several bioinformatic screens (Wilderman *et al.*, 2004; Livny *et al.*, 2006; Gonzalez *et al.*, 2008; Sonnleitner *et al.*, 2008), a shotgun cloning approach (Sonnleitner *et al.*, 2008) and co-purification with protein RsmA (Heeb *et al.*, 2002; Valverde *et al.*, 2003) were employed to unravel novel sRNAs.

3.4.4.1 Prrf 1/2

Iron is one of the most important metals in nature. It serves as a cofactor of many enzymes, which are involved in metabolic pathways such as the tricarboxylic (TCA) cycle, respiration, DNA synthesis and the synthesis of metabolites. Moreover, iron acquisition is a central prerequisit for successful establishment of pathogens in mammalian hosts. In most Gram-negative bacteria the ferric uptake regulator Fur negatively controls the expression of

genes involved in iron acquisition and of iron-regulated genes involved in virulence (Hantke, 2001). Fur can also act indirectly *via* extra-cytoplasmatic sigma factors or the sRNA RyhB (Massé and Gottesman, 2002; Massé *et al.*, 2007). During iron starvation *E. coli* Fur becomes inactive and the sRNA RyhB, which is under negative control of Fur is expressed. In the presence of Hfq it binds to its target mRNAs and thereby blocks synthesis of iron-containing enzymes including superoxide dismutase (*sodB*) (Massé *et al.*, 2003). In this way the RyhB leads to iron sparing in the cell, which is required to ensure cell maintainance (Jacques *et al.*, 2006). The *P. aeruginosa* intergenic regions were queried for Fur boxes followed by an up to 200 nt long spacer and a Rho-independent terminator. In this way two RyhB orthologs (Prrf1 and Prrf2) were discovered (Wilderman *et al.*, 2004). They are located in tandem, share 95% identity and each of them is preceded by a Fur-box (Wilderman *et al.*, 2004). A model for their proposed function is shown in Fig. 8.

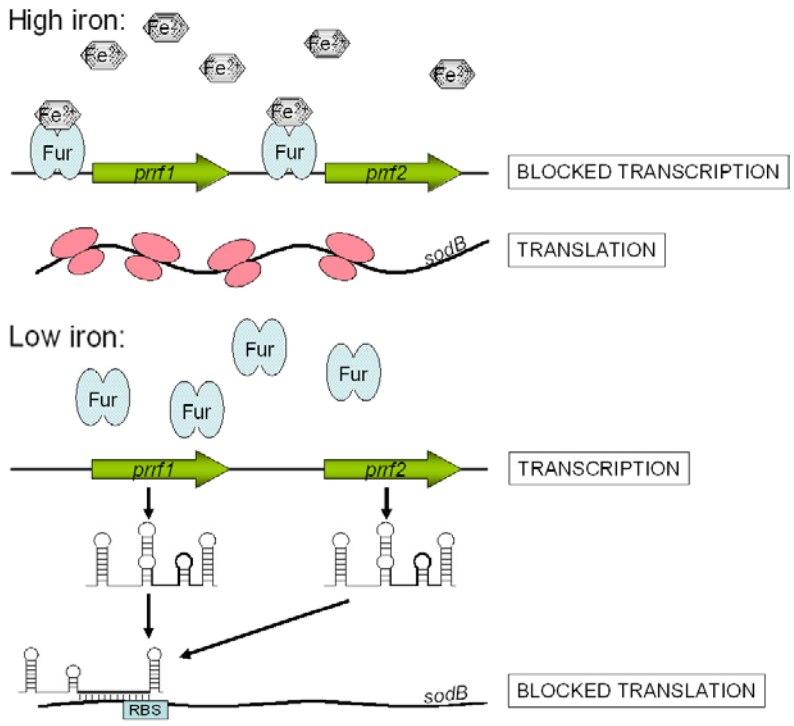


Fig. 8. PrrF expression depends on iron levels and controls translation of iron-containig enzymes, such as superoxide dismutase (*sodB*). Under iron replete conditions the Fur homo-dimer binds Fe^{2+} ions. Active Fe^{2+}-Fur binds to target mRNAs (e. g. *prrf1/2*) and blocks transcription. In the absence of Prrf1/2 mRNA targets are translated. When iron is scarce Fur becomes inactive and the Prrf1/2 sRNAs are transcribed. Binding of Prrf1/2 to regions in close proximity to the RBS of target mRNAs blocks translation.

Moreover, two putative *prrF* sequence homologs were found in *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas syringae*, but they are not located in tandem as in *P. aeruginosa* (Wilderman *et al.*, 2004). Microarray analysis revealed a large number of canditate Prrf-regulated genes. Among them were several genes involved in iron storage and oxidative stress protection, but the majority of Prrf-repressed genes appears to encode enzymes that participate in aerobic and anaerobic metabolism (Vasil, 2007; Oglesby *et al.*, 2008). Several of these functions are involved in the TCA cycle (aconitase A, aconitase B, succinate dehydrogenase), which have been also previously identified as being repressed by RyhB in *E. coli* (Massé and Gottesman, 2002; Massé *et al.*, 2005). Moreover, Oglesby *et. al.* (2008) showed that Prrf represses genes required for the degradation of anthranilate (*antABC* and *catBCA*), a precursor of the *Pseudomonas* quinolone quorum sensing signal (PQS). They proposed that repression of *antABC* could occur *via* direct interaction of Prrf with the mRNA encoding both AntA and its activator, AntR (Oglesby *et al.*, 2008). In this way iron could affect quorum sensing through regulation of PQS production. However, up to now no direct base-pairing interaction of Prrf1/2 with potential target mRNAs has been experimentally verified.

3.4.4.2 RgsA

The *P. aeruginosa* sRNA RgsA (regulation by GacA and stress; 1698 (Gonzalez *et al.*, 2008); P16 (Livny *et al.*, 2006)) was identified in two bioinformatic screens and is expressed during stationary phase of growth. RgsA expression is directly dependent on the stationary sigma factor RpoS and is probably indirectly activated by GacA. Further experiments revealed a decreased survival of RgsA mutants under oxidative stress (Gonzalez *et al.*, 2008). Its sequence is strongly conserved in other pseudomonads and it contains a GGA motif, which is typical for RsmA binding sRNAs, but it appears to be unable to sequester RsmA or RsmE (Gonzalez *et al.*, 2008).

3.4.4.3 PhrS and PhrD

The sRNAs PhrD and PhrS (P20 in Livny *et al.*, 2006; 1887 in Gonzales *et al.*, 2008) detected by RNomics after coimmunoprecipitation of RNAs with Hfq were verified by Northern-blot analysis. They encode 72 and 212 nt long RNAs, respectively (Sonnleitner *et al.*, 2008). Both bind to Hfq. Both of them show reduced steady state levels in an *hfq*- mutant, but their half-lifes are not affected, which suggests that Hfq is somehow required for their transcription. Several efforts to over-express PhrD failed, which could suggest that over-expression is lethal (Sonnleitner *et al.*, 2008). Plasmid-mediated over-expression of *phrS*, which was followed by a proteome analysis revealed GroEL, OprD and PA5153 (putative periplasmic binding protein) as potential targets of PhrS (Sonnleitner *et al.*, 2008). Inspection of the PhrS sequence disclosed an ORF encoding a 37 aa peptide, which was shown to be translated, but the function remains to be elucidated (Sonnleitner *et al.*, 2008). Preliminary data indicate that it is membrane associated (Sonnleitner *et al.*, unpublished). PhrS is induced upon hypoxia and requires the oxygen-responsive regulator ANR. *In silico* analyses together with transcriptome profiling studies upon overproduction of PhrS indicated that the sRNA targets the *pqsR* (*mvfR*) transcript, encoding an upstream transcriptional regulator involved in the pathway leading to synthesis of quinolone signal molecules and the virulence factor pyocyanin. Genetic studies suggested a novel mode of regulation by a sRNA, whereby PhrS activates a short open reading frame located upstream of *pqsR*, which in turn stimulates *pqsR* translation (Sonnleitner *et al.*, unpublished).

3.4.4.4 CRISPR

By using the CRISPRfinder webtool (Grissa *et al.*, 2007) CRISPR elements were located in several sequenced pseudomonads (*P. aeruginosa* UCBPP-PA14/ LESB58/ PA7, *P. entomophila* L48, *P. fluorescens* Pf-5, *P. mendocina* ymp, P. *putida* F1/ KT2440/ W619, *P stutzeri* A1501, P. syringae pv. phaseolicola 1448A/ pv. syringae B728a) harbour CRISPR similar sequences. However, no CRISPR were detected in *P. aeruginosa* PAO1, *P fluorescens*

PfO-1 and SPW25, *P. putida* GB-1 and in *P. synringae* pv. tomato str. DC3000. It was shown in *P. aeruginosa* PA14 that lysogenic infection with the phage DMS3 inhibits biofilm formation as well as swarming motility and that this inhibition requires the CRISPR locus in the host (Zegans *et al.*, 2009). The authors hypothesized that the bacterium might "quarantine" itself from biofilms to reduce the risk of infecting the larger community. Alternatively, inhibition of biofilm formation and swarming could provide a survival advantage to phage DMS3 (Zegans *et al.*, 2009). The CRISPR locus in *P. aeruginosa* PA14 is flanked by *cas* genes. Structural analysis of Cas1 revealed that it is a metal-dependent DNA specific endonuclease and probably involved in processing foreign DNA (Wiedenheft *et al.*, 2009).

3.4.4.5 Uncharacterized *P. aeruginosa* sRNAs

In *P. aeruginosa* bioinformatic screens were mainly employed to discover sRNAs. One approach focussed on Rho-independent terminators within conserved intergenic regions (Livny *et al.*, 2006). This screen yielded 38 potential candidates 17 of which could be verified by Northern-blot analysis. In addition, all four known aforementioned sRNAs (RsmZ, RsmY, Prrf1 and Prrf2) were detected in this screen.

Another screen (Gonzalez *et al.*, 2008) analyzed intergenic regions > 50 nt for sequence conservation among pseudomonads and subjected them to QRNA analysis (Rivas and Eddy, 2001). Among the 162 intergenic regions 32 contained tRNA and rRNA. Out of the remaining 130, for 49 a Rho-independent terminator and a potential promoter element was dicernable. 14 sRNAs were reproducibly detected by Northern-blot analysis. Among these were the previously described RNA of RNase P, 4.5S RNA, Prrf1/2 and three RNAs, which were detected before by Livny *et al.* (2006). In conclusion, this approach resulted in 8 novel sRNAs.

In our lab the bioinformatic tool RNAz was applied (Washietl *et al.*, 2005), which relies on the evolutionary conservation of RNA structure from a multiple sequence alignment. The

algorithm is based on the notion that structured RNAs fold into more stable secondary structures than the genomic background sequence of the same compostion (Sonnleitner *et al.*, 2008). This approach revealed seven sRNA loci, of which two were shown to be transcribed (Sonnleitner *et al.*, 2008). In addition, we used a shotgun-cloning approach in combination with Hfq co-immunopreciptation. This approach revealed three novel sRNA candidates PhrD, PhrX and PhrY (Sonnleitner *et al.*, 2008). In addition, tmRNA and two RNAs (PhrS, PhrW) identified by Livny *et al.* (2006) (P20, P28) and Gonzalez *et al*. (2008) (1887, 2510) were as well among the candidates. Moreover, RsmY, which is known to interact with Hfq (Sonnleitner *et al.*, 2006) was repeatedly present in the cDNA library (Sonnleitner *et al.*, 2008). In total 28 sRNAs have been detected in *P. aeruginosa* by Northern-blot analysis, the functions of which remain unknown.

3.5 Aim of the work

The aim of this PhD-thesis was to detect and characterize novel *P. aeruginosa* sRNAs. The search approaches concentrated on sRNAs, which act through base-pairing and thereby modulate translation and/or stability of messenger RNAs. First, bioinformatic screens as well as a shotgun cloning approach in combination with an Hfq co-immunoprecipitation were used to discover novel sRNA candidates. The sRNAs PaeII and PaeIII were predicted by a bioinformatic screen and their expression was verified by Northern-blot analysis. Second, proteome and transcriptome analyses were employed to identify target genes. Third, it was attempted to elucidate the mechanism through which the sRNAs adjust gene expression. The research focussed primarily on sRNAs that are involved in the regulation of virulence genes.

3.6 References

Akama, T., K. Suzuki, K. Tanigawa, A. Kawashima, H. Wu, N. Nakata, Y. Osana, Y. Sakakibara and N. Ishii (2009). "Whole-genome tiling array analysis of *Mycobacterium leprae* RNA reveals high expression of pseudogenes and noncoding regions." J Bacteriol **191**(10): 3321-7.

Altuvia, S. (2007). "Identification of bacterial small non-coding RNAs: experimental approaches." Curr Opin Microbiol **10**(3): 257-61.

Antal, M., V. Bordeau, V. Douchin and B. Felden (2005). "A small bacterial RNA regulates a putative ABC transporter." J Biol Chem **280**(9): 7901-8.

Azam, T. A. and A. Ishihama (1999). "Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity." J Biol Chem **274**(46): 33105-13.

Babitzke, P. and T. Romeo (2007). "CsrB sRNA family: sequestration of RNA-binding regulatory proteins." Curr Opin Microbiol **10**(2): 156-63.

Barrangou, R., C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero and P. Horvath (2007). "CRISPR provides acquired resistance against viruses in prokaryotes." Science **315**(5819): 1709-12.

Barrick, J. E., N. Sudarsan, Z. Weinberg, W. L. Ruzzo and R. R. Breaker (2005). "6S RNA is a widespread regulator of eubacterial RNA polymerase that resembles an open promoter." RNA **11**(5): 774-84.

Bouvier, M., C. M. Sharma, F. Mika, K. H. Nierhaus and J. Vogel (2008). "Small RNA binding to 5' mRNA coding region inhibits translational initiation." Mol Cell **32**(6): 827-37.

Brantl, S. (2007). "Regulatory mechanisms employed by *cis*-encoded antisense RNAs." Curr Opin Microbiol **10**(2): 102-9.

Brencic, A., K. A. McFarland, H. R. McManus, S. Castang, I. Mogno, S. L. Dove and S. Lory (2009). "The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs." Mol Microbiol **73**(3): 434-45.

Brescia, C. C., P. J. Mikulecky, A. L. Feig and D. D. Sledjeski (2003). "Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure." RNA **9**(1): 33-43.

Chant, E. L. and D. K. Summers (2007). "Indole signalling contributes to the stable maintenance of *Escherichia coli* multicopy plasmids." Mol Microbiol **63**(1): 35-43.

Christiansen, J. K., J. S. Nielsen, T. Ebersbach, P. Valentin-Hansen, L. Sogaard-Andersen and B. H. Kallipolitis (2006). "Identification of small Hfq-binding RNAs in *Listeria monocytogenes*." RNA **12**(7): 1383-96.

Cooley, M., S. R. Chhabra and P. Williams (2008). "N-Acylhomoserine lactone-mediated quorum sensing: a twist in the tail and a blow for host immunity." Chem Biol **15**(11): 1141-7.

Darfeuille, F., C. Unoson, J. Vogel and E. G. Wagner (2007). "An antisense RNA inhibits translation by competing with standby ribosomes." Mol Cell **26**(3): 381-92.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton and E. P. Greenberg (1998). "The involvement of cell-to-cell signals in the development of a bacterial biofilm." Science **280**(5361): 295-8.

De Lay, N. and S. Gottesman (2009). "The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior." J Bacteriol **191**(2): 461-76.

Ding, Y., B. M. Davis and M. K. Waldor (2004). "Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression." Mol Microbiol **53**(1): 345-54.

Douchin, V., C. Bohn and P. Bouloc (2006). "Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*." J Biol Chem **281**(18): 12253-9.

Feldman, M., R. Bryan, S. Rajan, L. Scheffler, S. Brunnert, H. Tang and A. Prince (1998). "Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection." Infect Immun **66**(1): 43-51.

Fierro-Monti, I. P., S. J. Reid and D. R. Woods (1992). "Differential expression of a *Clostridium acetobutylicum* antisense RNA: implications for regulation of glutamine synthetase." J Bacteriol **174**(23): 7642-7.

Folichon, M., V. Arluison, O. Pellegrini, E. Huntzinger, P. Regnier and E. Hajnsdorf (2003). "The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation." Nucleic Acids Res **31**(24): 7302-10.

Folichon, M., F. Allemand, P. Regnier and E. Hajnsdorf (2005). "Stimulation of poly(A) synthesis by *Escherichia coli* poly(A)polymerase I is correlated with Hfq binding to poly(A) tails." FEBS J **272**(2): 454-63.

Franze de Fernandez, M. T., L. Eoyang and J. T. August (1968). "Factor fraction required for the synthesis of bacteriophage Qbeta-RNA." Nature **219**(154): 588-90.

Fröhlich, K. S. and J. Vogel (2009). "Activation of gene expression by small RNA." Curr Opin Microbiol **12**(6): 674-82.

Gambello, M. J., S. Kaye and B. H. Iglewski (1993). "LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression." Infect Immun **61**(4): 1180-4.

Giangrossi, M., G. Prosseda, C. N. Tran, A. Brandi, B. Colonna and M. Falconi (2010). "A novel antisense RNA regulates at transcriptional level the virulence gene *icsA* of *Shigella flexneri*." Nucleic Acids Res.

Gonzalez, N., S. Heeb, C. Valverde, E. Kay, C. Reimmann, T. Junier and D. Haas (2008). "Genome-wide search reveals a novel GacA-regulated small RNA in *Pseudomonas* species." BMC Genomics **9**: 167.

Goodman, A. L., B. Kulasekara, A. Rietsch, D. Boyd, R. S. Smith and S. Lory (2004). "A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*." Dev Cell **7**(5): 745-54.

Goodman, A. L., M. Merighi, M. Hyodo, I. Ventre, A. Filloux and S. Lory (2009). "Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen." Genes Dev **23**(2): 249-59.

Görke, B. and J. Stülke (2008). "Carbon catabolite repression in bacteria: many ways to make the most out of nutrients." Nat Rev Microbiol **6**(8): 613-24.

Görke, B. and J. Vogel (2008). "Noncoding RNA control of the making and breaking of sugars." Genes Dev **22**(21): 2914-25.

Gottesman, S. (2004). "The small RNA regulators of *Escherichia coli*: roles and mechanisms." Annu Rev Microbiol **58**: 303-28.

Gottesman, S. (2005). "Micros for microbes: non-coding regulatory RNAs in bacteria." Trends Genet **21**(7): 399-404.

Grissa, I., G. Vergnaud and C. Pourcel (2007). "CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats." Nucleic Acids Res **35**(Web Server issue): W52-7.

Gudapaty, S., K. Suzuki, X. Wang, P. Babitzke and T. Romeo (2001). "Regulatory interactions of Csr components: the RNA binding protein CsrA activates *csrB* transcription in *Escherichia coli*." J Bacteriol **183**(20): 6017-27.

Hajnsdorf, E. and P. Regnier (2000). "Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I." Proc Natl Acad Sci U S A **97**(4): 1501-5.

Hantke, K. (2001). "Iron and metal regulation in bacteria." Curr Opin Microbiol **4**(2): 172-7.

Hauser, A. R. (2009). "The type III secretion system of *Pseudomonas aeruginosa*: infection by injection." Nat Rev Microbiol **7**(9): 654-65.

Heeb, S., C. Blumer and D. Haas (2002). "Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0." J Bacteriol **184**(4): 1046-56.

Heeb, S., C. Valverde, C. Gigot-Bonnefoy and D. Haas (2005). "Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHA0." FEMS Microbiol Lett **243**(1): 251-8.

Heurlier, K., V. Denervaud, G. Pessi, C. Reimmann and D. Haas (2003). "Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1." J Bacteriol **185**(7): 2227-35.

Hu, Z., A. Zhang, G. Storz, S. Gottesman and S. H. Leppla (2006). "An antibody-based microarray assay for small RNA detection." Nucleic Acids Res **34**(7): e52.

Humair, B., N. Gonzalez, D. Mossialos, C. Reimmann and D. Haas (2009). "Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0." ISME J **3**(8): 955-65.

Jacques, J. F., S. Jang, K. Prevost, G. Desnoyers, M. Desmarais, J. Imlay and E. Massé (2006). "RyhB small RNA modulates the free intracellular iron pool and is essential for normal growth during iron limitation in *Escherichia coli*." Mol Microbiol **62**(4): 1181-90.

James, B. D., G. J. Olsen, J. S. Liu and N. R. Pace (1988). "The secondary structure of ribonuclease P RNA, the catalytic element of a ribonucleoprotein enzyme." Cell **52**(1): 19-26.

Janzon, L., S. Lofdahl and S. Arvidson (1989). "Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*." Mol Gen Genet **219**(3): 480-5.

Johansen, J., A. A. Rasmussen, M. Overgaard and P. Valentin-Hansen (2006). "Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins." J Mol Biol **364**(1): 1-8.

Jousselin, A., L. Metzinger and B. Felden (2009). "On the facultative requirement of the bacterial RNA chaperone, Hfq." Trends Microbiol **17**(9): 399-405.

Juhas, M., L. Eberl and B. Tümmler (2005). "Quorum sensing: the power of cooperation in the world of *Pseudomonas*." Environ Microbiol **7**(4): 459-71.

Kaberdin, V. R. and U. Bläsi (2006). "Translation initiation and the fate of bacterial mRNAs." FEMS Microbiol Rev **30**(6): 967-79.

Kajitani, M., A. Kato, A. Wada, Y. Inokuchi and A. Ishihama (1994). "Regulation of the *Escherichia coli hfq* gene encoding the host factor for phage Q beta." J Bacteriol **176**(2): 531-4.

Kalamorz, F., B. Reichenbach, W. März, B. Rak and B. Görke (2007). "Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA GlmZ and involves the novel protein YhbJ in *Escherichia coli*." Mol Microbiol **65**(6): 1518-33.

Kay, E., C. Dubuis and D. Haas (2005). "Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0." Proc Natl Acad Sci U S A **102**(47): 17136-41.

Kay, E., B. Humair, V. Denervaud, K. Riedel, S. Spahr, L. Eberl, C. Valverde and D. Haas (2006). "Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*." J Bacteriol **188**(16): 6026-33.

Kazantsev, A. V. and N. R. Pace (2006). "Bacterial RNase P: a new view of an ancient enzyme." Nat Rev Microbiol **4**(10): 729-40.

Keiler, K. C. (2007). "Physiology of tmRNA: what gets tagged and why?" Curr Opin Microbiol **10**(2): 169-75.

Kilic, T., S. Thore and D. Suck (2005). "Crystal structure of an archaeal Sm protein from *Sulfolobus solfataricus*." Proteins **61**(3): 689-93.

Lapouge, K., E. Sineva, M. Lindell, K. Starke, C. S. Baker, P. Babitzke and D. Haas (2007). "Mechanism of *hcnA* mRNA recognition in the Gac/Rsm signal transduction pathway of *Pseudomonas fluorescens*." Mol Microbiol **66**(2): 341-56.

Lapouge, K., M. Schubert, F. H. Allain and D. Haas (2008). "Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour." Mol Microbiol **67**(2): 241-53.

Laskowski, M. A., E. Osborn and B. I. Kazmierczak (2004). "A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*." Mol Microbiol **54**(4): 1090-103.

Lease, R. A., M. E. Cusick and M. Belfort (1998). "Riboregulation in *Escherichia coli*: DsrA RNA acts by RNA:RNA interactions at multiple loci." Proc Natl Acad Sci U S A **95**(21): 12456-61.

Lenz, D. H., K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen and B. L. Bassler (2004). "The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*." Cell **118**(1): 69-82.

Link, T. M., P. Valentin-Hansen and R. G. Brennan (2009). "Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA." Proc Natl Acad Sci U S A **106**(46): 19292-7.

Liu, J. M., J. Livny, M. S. Lawrence, M. D. Kimball, M. K. Waldor and A. Camilli (2009). "Experimental discovery of sRNAs in *Vibrio cholerae* by direct cloning, 5S/tRNA depletion and parallel sequencing." Nucleic Acids Res **37**(6): e46.

Liu, M. Y., G. Gui, B. Wei, J. F. Preston, 3rd, L. Oakford, U. Yuksel, D. P. Giedroc and T. Romeo (1997). "The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*." J Biol Chem **272**(28): 17502-10.

Livny, J., A. Brencic, S. Lory and M. K. Waldor (2006). "Identification of 17 *Pseudomonas aeruginosa* sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2." Nucleic Acids Res **34**(12): 3484-93.

Lorenz, C., F. von Pelchrzim and R. Schroeder (2006). "Genomic systematic evolution of ligands by exponential enrichment (Genomic SELEX) for the identification of protein-binding RNAs independent of their expression levels." Nat Protoc **1**(5): 2204-12.

Lorenz, C., T. Gesell, B. Zimmermann, U. Schoeberl, I. Bilusic, L. Rajkowitsch, C. Waldsich, A. von Haeseler and R. Schroeder (2010). "Genomic SELEX for Hfq-binding RNAs identifies genomic aptamers predominantly in antisense transcripts." Nucleic Acids Res.

Lucchetti-Miganeh, C., E. Burrowes, C. Baysse and G. Ermel (2008). "The post-transcriptional regulator CsrA plays a central role in the adaptation of bacterial pathogens to different stages of infection in animal hosts." Microbiology **154**(Pt 1): 16-29.

Mandin, P. and S. Gottesman (2009). "A genetic approach for finding small RNAs regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system." Mol Microbiol **72**(3): 551-65.

Massé, E. and S. Gottesman (2002). "A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*." Proc Natl Acad Sci U S A **99**(7): 4620-5.

Massé, E., F. E. Escorcia and S. Gottesman (2003). "Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*." Genes Dev **17**(19): 2374-83.

Massé, E., C. K. Vanderpool and S. Gottesman (2005). "Effect of RyhB small RNA on global iron use in *Escherichia coli*." J Bacteriol **187**(20): 6962-71.

Massé, E., H. Salvail, G. Desnoyers and M. Arguin (2007). "Small RNAs controlling iron metabolism." Curr Opin Microbiol **10**(2): 140-5.

Mathee, K., G. Narasimhan, C. Valdes, X. Qiu, J. M. Matewish, M. Koehrsen, A. Rokas, C. N. Yandava, R. Engels, E. Zeng, R. Olavarietta, M. Doud, R. S. Smith, P. Montgomery, J. R. White, P. A. Godfrey, C. Kodira, B. Birren, J. E. Galagan and S. Lory (2008). "Dynamics of *Pseudomonas aeruginosa* genome evolution." Proc Natl Acad Sci U S A **105**(8): 3100-5.

McNealy, T. L., V. Forsbach-Birk, C. Shi and R. Marre (2005). "The Hfq homolog in *Legionella pneumophila* demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA." J Bacteriol **187**(4): 1527-32.

Mikulecky, P. J., M. K. Kaw, C. C. Brescia, J. C. Takach, D. D. Sledjeski and A. L. Feig (2004). "*Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs." Nat Struct Mol Biol **11**(12): 1206-14.

Mohanty, B. K., V. F. Maples and S. R. Kushner (2004). "The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*." Mol Microbiol **54**(4): 905-20.

Moll, I., T. Afonyushkin, O. Vytvytska, V. R. Kaberdin and U. Bläsi (2003a). "Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs." RNA **9**(11): 1308-14.

Moll, I., D. Leitsch, T. Steinhauser and U. Bläsi (2003b). "RNA chaperone activity of the Sm-like Hfq protein." EMBO Rep **4**(3): 284-9.

Møller, T., T. Franch, P. Hojrup, D. R. Keene, H. P. Bachinger, R. G. Brennan and P. Valentin-Hansen (2002). "Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction." Mol Cell **9**(1): 23-30.

Mondragon, V., B. Franco, K. Jonas, K. Suzuki, T. Romeo, O. Melefors and D. Georgellis (2006). "pH-dependent activation of the BarA-UvrY two-component system in *Escherichia coli*." J Bacteriol **188**(23): 8303-6.

Morfeldt, E., D. Taylor, A. von Gabain and S. Arvidson (1995). "Activation of alpha-toxin translation in *Staphylococcus aureus* by the *trans*-encoded antisense RNA, RNAIII." EMBO J **14**(18): 4569-77.

Nakae, T. (1995). "Role of membrane permeability in determining antibiotic resistance in *Pseudomonas aeruginosa*." Microbiol Immunol **39**(4): 221-9.

Nakao, H., H. Watanabe, S. Nakayama and T. Takeda (1995). "*yst* gene expression in *Yersinia enterocolitica* is positively regulated by a chromosomal region that is highly homologous to *Escherichia coli* host factor 1 gene (*hfq*)." Mol Microbiol **18**(5): 859-65.

Nielsen, J. S., A. Boggild, C. B. Andersen, G. Nielsen, A. Boysen, D. E. Brodersen and P. Valentin-Hansen (2007). "An Hfq-like protein in archaea: crystal structure and functional characterization of the Sm protein from *Methanococcus jannaschii*." RNA **13**(12): 2213-23.

Nielsen, J. S., L. K. Lei, T. Ebersbach, A. S. Olsen, J. K. Klitgaard, P. Valentin-Hansen and B. H. Kallipolitis (2009). "Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*." Nucleic Acids Res.

Nikaido, H. (1996). "Multidrug efflux pumps of gram-negative bacteria." J Bacteriol **178**(20): 5853-9.

Nikulin, A., E. Stolboushkina, A. Perederina, I. Vassilieva, U. Bläsi, I. Moll, G. Kachalova, S. Yokoyama, D. Vassylyev, M. Garber and S. Nikonov (2005). "Structure of *Pseudomonas aeruginosa* Hfq protein." Acta Crystallogr D Biol Crystallogr **61**(Pt 2): 141-6.

Oglesby, A. G., J. M. Farrow, 3rd, J. H. Lee, A. P. Tomaras, E. P. Greenberg, E. C. Pesci and M. L. Vasil (2008). "The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing." J Biol Chem **283**(23): 15558-67.

Papenfort, K., N. Said, T. Welsink, S. Lucchini, J. C. Hinton and J. Vogel (2009). "Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA." Mol Microbiol **74**(1): 139-58.

Papenfort, K. and J. Vogel (2009). "Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level." Res Microbiol **160**(4): 278-87.

Passalacqua, K. D., A. Varadarajan, B. D. Ondov, D. T. Okou, M. E. Zwick and N. H. Bergman (2009). "Structure and complexity of a bacterial transcriptome." J Bacteriol **191**(10): 3203-11.

Pfeiffer, V., K. Papenfort, S. Lucchini, J. C. Hinton and J. Vogel (2009). "Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation." Nat Struct Mol Biol **16**(8): 840-6.

Pichon, C. and B. Felden (2008). "Small RNA gene identification and mRNA target predictions in bacteria." Bioinformatics **24**(24): 2807-13.

Piddock, L. J. (2006). "Multidrug-resistance efflux pumps - not just for resistance." Nat Rev Microbiol **4**(8): 629-36.

Rasmussen, A. A., M. Eriksen, K. Gilany, C. Udesen, T. Franch, C. Petersen and P. Valentin-Hansen (2005). "Regulation of *ompA* mRNA stability: the role of a small regulatory RNA in growth phase-dependent control." Mol Microbiol **58**(5): 1421-9.

Reichenbach, B., A. Maes, F. Kalamorz, E. Hajnsdorf and B. Görke (2008). "The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*." Nucleic Acids Res **36**(8): 2570-80.

Reimmann, C., C. Valverde, E. Kay and D. Haas (2005). "Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0." J Bacteriol **187**(1): 276-85.

Repoila, F., N. Majdalani and S. Gottesman (2003). "Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm." Mol Microbiol **48**(4): 855-61.

Repoila, F. and F. Darfeuille (2009). "Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects." Biol Cell **101**(2): 117-31.

Resch, A., T. Afonyushkin, T. B. Lombo, K. J. McDowall, U. Bläsi and V. R. Kaberdin (2008). "Translational activation by the noncoding RNA DsrA involves alternative RNase III processing in the *rpoS* 5'-leader." RNA **14**(3): 454-9.

Rivas, E. and S. R. Eddy (2001). "Noncoding RNA gene detection using comparative sequence analysis." BMC Bioinformatics **2**: 8.

Robertson, G. T. and R. M. Roop, Jr. (1999). "The *Brucella abortus* host factor I (HF-I) protein contributes to stress resistance during stationary phase and is a major determinant of virulence in mice." Mol Microbiol **34**(4): 690-700.

Sauter, C., J. Basquin and D. Suck (2003). "Sm-like proteins in Eubacteria: the crystal structure of the Hfq protein from *Escherichia coli*." Nucleic Acids Res **31**(14): 4091-8.

Schubert, M., K. Lapouge, O. Duss, F. C. Oberstrass, I. Jelesarov, D. Haas and F. H. Allain (2007). "Molecular basis of messenger RNA recognition by the specific bacterial repressing clamp RsmA/CsrA." Nat Struct Mol Biol **14**(9): 807-13.

Schumacher, M. A., R. F. Pearson, T. Møller, P. Valentin-Hansen and R. G. Brennan (2002). "Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein." EMBO J **21**(13): 3546-56.

Sharma, C. M., F. Darfeuille, T. H. Plantinga and J. Vogel (2007). "A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites." Genes Dev **21**(21): 2804-17.

Sharma, C. M. and J. Vogel (2009). "Experimental approaches for the discovery and characterization of regulatory small RNA." Curr Opin Microbiol **12**(5): 536-46.

Silvaggi, J. M., J. B. Perkins and R. Losick (2005). "Small untranslated RNA antitoxin in *Bacillus subtilis*." J Bacteriol **187**(19): 6641-50.

Sittka, A., V. Pfeiffer, K. Tedin and J. Vogel (2007). "The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*." Mol Microbiol **63**(1): 193-217.

Sledjeski, D. D., C. Whitman and A. Zhang (2001). "Hfq is necessary for regulation by the untranslated RNA DsrA." J Bacteriol **183**(6): 1997-2005.

Sonnleitner, E., S. Hagens, F. Rosenau, S. Wilhelm, A. Habel, K. E. Jäger and U. Bläsi (2003). "Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1." Microb Pathog **35**(5): 217-28.

Sonnleitner, E., M. Schuster, T. Sorger-Domenigg, E. P. Greenberg and U. Bläsi (2006). "Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*." Mol Microbiol **59**(5): 1542-58.

Sonnleitner, E., T. Sorger-Domenigg, M. J. Madej, S. Findeiss, J. Hackermüller, A. Hüttenhofer, P. F. Stadler, U. Bläsi and I. Moll (2008). "Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools." Microbiology **154**(Pt 10): 3175-87.

Sonnleitner, E., L. Abdou and D. Haas (2009). "Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*." Proc Natl Acad Sci U S A.

Sonnleitner, E., N. Gonzalez and D. Haas (2010). Small RNAs of *Pseudomonas* spp. Pseudomonas. J.-L. Ramos and A. Filloux. Netherlands, Springer. **6: Molecular Microbiology, Infection and Biodiversity:** 3-28.

Sorek, R., V. Kunin and P. Hugenholtz (2008). "CRISPR--a widespread system that provides acquired resistance against phages in bacteria and archaea." Nat Rev Microbiol **6**(3): 181-6.

Sorger-Domenigg, T., E. Sonnleitner, V. R. Kaberdin and U. Bläsi (2007). "Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY." Biochem Biophys Res Commun **352**(3): 769-73.

Steglich, C., M. E. Futschik, D. Lindell, B. Voss, S. W. Chisholm and W. R. Hess (2008). "The challenge of regulation in a minimal photoautotroph: non-coding RNAs in *Prochlorococcus*." PLoS Genet **4**(8): e1000173.

Sukhodolets, M. V. and S. Garges (2003). "Interaction of *Escherichia coli* RNA polymerase with the ribosomal protein S1 and the Sm-like ATPase Hfq." Biochemistry **42**(26): 8022-34.

Sun, X., I. Zhulin and R. M. Wartell (2002). "Predicted structure and phyletic distribution of the RNA-binding protein Hfq." Nucleic Acids Res **30**(17): 3662-71.

Sun, X. and R. M. Wartell (2006). "*Escherichia coli* Hfq binds A18 and DsrA domain II with similar 2:1 Hfq6/RNA stoichiometry using different surface sites." Biochemistry **45**(15): 4875-87.

Suzuki, K., X. Wang, T. Weilbacher, A. K. Pernestig, O. Melefors, D. Georgellis, P. Babitzke and T. Romeo (2002). "Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*." J Bacteriol **184**(18): 5130-40.

Suzuki, K., P. Babitzke, S. R. Kushner and T. Romeo (2006). "Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E." Genes Dev **20**(18): 2605-17.

Tjaden, B., R. M. Saxena, S. Stolyar, D. R. Haynor, E. Kolker and C. Rosenow (2002). "Transcriptome analysis of *Escherichia coli* using high-density oligonucleotide probe arrays." Nucleic Acids Res **30**(17): 3732-8.

Tjaden, B., S. S. Goodwin, J. A. Opdyke, M. Guillier, D. X. Fu, S. Gottesman and G. Storz (2006). "Target prediction for small, noncoding RNAs in bacteria." Nucleic Acids Res **34**(9): 2791-802.

Toder, D. S., S. J. Ferrell, J. L. Nezezon, L. Rust and B. H. Iglewski (1994). "*lasA* and *lasB* genes of *Pseudomonas aeruginosa*: analysis of transcription and gene product activity." Infect Immun **62**(4): 1320-7.

Toledo-Arana, A., O. Dussurget, G. Nikitas, N. Sesto, H. Guet-Revillet, D. Balestrino, E. Loh, J. Gripenland, T. Tiensuu, K. Vaitkevicius, M. Barthelemy, M. Vergassola, M. A. Nahori, G. Soubigou, B. Regnault, J. Y. Coppee, M. Lecuit, J. Johansson and P. Cossart (2009). "The *Listeria* transcriptional landscape from saprophytism to virulence." Nature **459**(7249): 950-6.

Trotochaud, A. E. and K. M. Wassarman (2005). "A highly conserved 6S RNA structure is required for regulation of transcription." Nat Struct Mol Biol **12**(4): 313-9.

Tsui, H. C., H. C. Leung and M. E. Winkler (1994). "Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12." Mol Microbiol **13**(1): 35-49.

Tsui, H. C., G. Feng and M. E. Winkler (1997). "Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12." J Bacteriol **179**(23): 7476-87.

Udekwu, K. I., F. Darfeuille, J. Vogel, J. Reimegard, E. Holmqvist and E. G. Wagner (2005). "Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA." Genes Dev **19**(19): 2355-66.

Ullmann, A. (1996). "Catabolite repression: a story without end." Res Microbiol **147**(6-7): 455-8.

Urban, J. H. and J. Vogel (2007). "Translational control and target recognition by *Escherichia coli* small RNAs *in vivo*." Nucleic Acids Res **35**(3): 1018-37.

Urban, J. H. and J. Vogel (2008). "Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation." PLoS Biol **6**(3): e64.

Urban, J. H. and J. Vogel (2009). "A green fluorescent protein (GFP)-based plasmid system to study post-transcriptional control of gene expression *in vivo*." Methods Mol Biol **540**: 301-19.

Urbanowski, M. L., L. T. Stauffer and G. V. Stauffer (2000). "The *gcvB* gene encodes a small untranslated RNA involved in expression of the dipeptide and oligopeptide transport systems in *Escherichia coli*." Mol Microbiol **37**(4): 856-68.

Valentin-Hansen, P., M. Eriksen and C. Udesen (2004). "The bacterial Sm-like protein Hfq: a key player in RNA transactions." Mol Microbiol **51**(6): 1525-33.

Valverde, C., S. Heeb, C. Keel and D. Haas (2003). "RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0." Mol Microbiol **50**(4): 1361-79.

Valverde, C., M. Lindell, E. G. Wagner and D. Haas (2004). "A repeated GGA motif is critical for the activity and stability of the riboregulator RsmY of *Pseudomonas fluorescens*." J Biol Chem **279**(24): 25066-74.

van Delden, C. and B. H. Iglewski (1998). "Cell-to-cell signaling and *Pseudomonas aeruginosa* infections." Emerg Infect Dis **4**(4): 551-60.

van der Oost, J., M. M. Jore, E. R. Westra, M. Lundgren and S. J. Brouns (2009). "CRISPR-based adaptive and heritable immunity in prokaryotes." Trends Biochem Sci **34**(8): 401-7.

Vasil, M. L. (2007). "How we learnt about iron acquisition in *Pseudomonas aeruginosa*: a series of very fortunate events." Biometals **20**(3-4): 587-601.

Vecerek, B., I. Moll and U. Bläsi (2005). "Translational autocontrol of the *Escherichia coli hfq* RNA chaperone gene." RNA **11**(6): 976-84.

Vecerek, B., I. Moll and U. Bläsi (2007). "Control of Fur synthesis by the non-coding RNA RyhB and iron-responsive decoding." EMBO J **26**(4): 965-75.

Vecerek, B., L. Rajkowitsch, E. Sonnleitner, R. Schroeder and U. Bläsi (2008). "The C-terminal domain of *Escherichia coli* Hfq is required for regulation." Nucleic Acids Res **36**(1): 133-43.

Ventre, I., A. L. Goodman, I. Vallet-Gely, P. Vasseur, C. Soscia, S. Molin, S. Bleves, A. Lazdunski, S. Lory and A. Filloux (2006). "Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes." Proc Natl Acad Sci U S A **103**(1): 171-6.

Vogel, D. W., R. K. Hartmann, J. C. Struck, N. Ulbrich and V. A. Erdmann (1987). "The sequence of the 6S RNA gene of *Pseudomonas aeruginosa*." Nucleic Acids Res **15**(11): 4583-91.

Vogel, J., V. Bartels, T. H. Tang, G. Churakov, J. G. Slagter-Jäger, A. Hüttenhofer and E. G. Wagner (2003). "RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria." Nucleic Acids Res **31**(22): 6435-43.

Vogel, J. and C. M. Sharma (2005). "How to find small non-coding RNAs in bacteria." Biol Chem **386**(12): 1219-38.

Vogel, J. and E. G. Wagner (2007). "Target identification of small noncoding RNAs in bacteria." Curr Opin Microbiol **10**(3): 262-70.

Vytvytska, O., J. S. Jakobsen, G. Balcunaite, J. S. Andersen, M. Baccarini and A. von Gabain (1998). "Host factor I, Hfq, binds to *Escherichia coli ompA* mRNA in a growth rate-dependent fashion and regulates its stability." Proc Natl Acad Sci U S A **95**(24): 14118-23.

Wadler, C. S. and C. K. Vanderpool (2007). "A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide." Proc Natl Acad Sci U S A **104**(51): 20454-9.

Washietl, S., I. L. Hofacker and P. F. Stadler (2005). "Fast and reliable prediction of noncoding RNAs." Proc Natl Acad Sci U S A **102**(7): 2454-9.

Wassarman, K. M., A. Zhang and G. Storz (1999). "Small RNAs in *Escherichia coli*." Trends Microbiol **7**(1): 37-45.

Wassarman, K. M. (2007). "6S RNA: a small RNA regulator of transcription." Curr Opin Microbiol **10**(2): 164-8.

Waters, L. S. and G. Storz (2009). "Regulatory RNAs in bacteria." Cell **136**(4): 615-28.

Weilbacher, T., K. Suzuki, A. K. Dubey, X. Wang, S. Gudapaty, I. Morozov, C. S. Baker, D. Georgellis, P. Babitzke and T. Romeo (2003). "A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*." Mol Microbiol **48**(3): 657-70.

Wick, M. J., A. N. Hamood and B. H. Iglewski (1990). "Analysis of the structure-function relationship of *Pseudomonas aeruginosa* exotoxin A." Mol Microbiol **4**(4): 527-35.

Wiedenheft, B., K. Zhou, M. Jinek, S. M. Coyle, W. Ma and J. A. Doudna (2009). "Structural basis for DNase activity of a conserved protein implicated in CRISPR-mediated genome defense." Structure **17**(6): 904-12.

Wilderman, P. J., N. A. Sowa, D. J. FitzGerald, P. C. FitzGerald, S. Gottesman, U. A. Ochsner and M. L. Vasil (2004). "Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis." Proc Natl Acad Sci U S A **101**(26): 9792-7.

Williams, K. P. and D. P. Bartel (1996). "Phylogenetic analysis of tmRNA secondary structure." RNA **2**(12): 1306-10.

Williams, P. and M. Camara (2009). "Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules." Curr Opin Microbiol **12**(2): 182-91.

Zegans, M. E., J. C. Wagner, K. C. Cady, D. M. Murphy, J. H. Hammond and G. A. O'Toole (2009). "Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of *Pseudomonas aeruginosa*." J Bacteriol **191**(1): 210-9.

Zhang, A., K. M. Wassarman, J. Ortega, A. C. Steven and G. Storz (2002). "The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs." Mol Cell **9**(1): 11-22.

Zhang, A., K. M. Wassarman, C. Rosenow, B. C. Tjaden, G. Storz and S. Gottesman (2003). "Global analysis of small RNA and mRNA targets of Hfq." Mol Microbiol **50**(4): 1111-24.

4 MANUSCRIPTS

4.1 Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools

Microbiology (2008), **154**, 3175–3187 DOI 10.1099/mic.0.2008/019703-0

Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools

Elisabeth Sonnleitner,[1] Theresa Sorger-Domenigg,[1] Monika J. Madej,[2] Sven Findeiss,[3] Jörg Hackermüller,[3] Alexander Hüttenhofer,[2] Peter F. Stadler,[3] Udo Bläsi[1] and Isabella Moll[1]

Correspondence
Isabella Moll
Isabella.Moll@univie.ac.at

[1]Max F. Perutz Laboratories, Department of Microbiology and Immunobiology, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria

[2]Innsbruck Biocenter, Division of Genomics and RNomics, Medical University Innsbruck, Fritz-Pregl Str. 3, A-6020 Innsbruck, Austria

[3]Bioinformatics Group, Department of Computer Science and Interdisciplinary Center for Bioinformatics, University of Leipzig, Härtelstraße 16-18, D-04107 Leipzig, Germany

Inactivation of the *Pseudomonas aeruginosa* (PAO1) *hfq* gene, encoding the Sm-like Hfq protein, resulted in pleiotropic effects that included an attenuated virulence. As regulation by Hfq often involves the action of small regulatory RNAs (sRNAs), we have used a shotgun cloning approach (RNomics) and bioinformatic tools to identify sRNAs in strain PAO1. For cDNA library construction, total RNA was extracted from PAO1 cultures either grown to stationary phase or exposed to human serum. The cDNA libraries were generated from small-sized RNAs of PAO1 after co-immunoprecipitation with Hfq. Of 400 sequenced cDNA clones, 11 mapped to intergenic regions. Band-shift assays and Northern blot analyses performed with two selected sRNAs confirmed that Hfq binds to and affects the steady-state levels of these RNAs. A proteome study performed upon overproduction of one sRNA, PhrS, implicated it in riboregulation. PhrS contains an ORF, and evidence for its translation is presented. In addition, based on surveys with structure-based bioinformatic tools, we provide an electronic compilation of putative sRNA and non-coding RNA genes of PAO1 based on their evolutionarily conserved structure.

Received 16 April 2008
Revised 27 June 2008
Accepted 4 July 2008

INTRODUCTION

During the last few years an increasing number of non-coding RNAs (ncRNAs) and small regulatory RNAs (sRNAs) has been described in different pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae* and *Pseudomonas aeruginosa* (strain PAO1) (Argaman *et al.*, 2001; Sittka *et al.*, 2007; Heurlier *et al.*, 2004; Lenz *et al.*, 2004; Wilderman *et al.*, 2004; Zhang *et al.*, 2003). Some of these sRNAs are involved in quorum sensing (QS) and bacterial pathogenesis, such as RsmY and RsmZ in PAO1 (Heurlier *et al.*, 2004; Sonnleitner *et al.*, 2006) and Qrr1-4 in *V. cholerae* (Lenz *et al.*, 2004). Another group of sRNAs modulates the stress response by regulating the expression of the alternative sigma factor σ^S (Repoila *et al.*, 2003), while others are important for iron metabolism (Massé & Gottesman, 2002; Wilderman *et al.*, 2004), catabolite regulation (Møller *et al.*, 2002) and for remodelling of the outer membrane (Guillier *et al.*, 2006). A large number of sRNAs has been shown to associate with Hfq and to require this protein for post-transcriptional regulation (Gottesman, 2005; Massé *et al.*, 2003). Part of this requirement can be explained by Hfq-mediated stabilization of some of the studied sRNAs, e.g. DsrA, Spot42 RNA, RyhB and RsmY (Møller *et al.*, 2002; Moll *et al.*, 2003a; Sledjeski *et al.*, 2001; Sonnleitner *et al.*, 2006), as well as by the RNA chaperone function of Hfq (Moll *et al.*, 2003b), which facilitates the interaction of sRNAs with their target mRNAs (Kawamoto *et al.*, 2006; Zhang *et al.*, 2002).

Hfq has been identified as a virulence factor in *Yersinia enterocolitica*, *Brucella abortus*, *Legionella pneumophila*, *Salmonella typhimurium* and *V. cholerae* (Sittka *et al.*, 2007; Ding *et al.*, 2004; McNealy *et al.*, 2005; Nakao *et al.*,

Abbreviations: ncRNA, non-coding RNA; QS, quorum sensing; sRNA, small regulatory RNA; SVM, support vector machine.

Three supplementary figures showing the genetic organization and predicted secondary structures of PhrX and PhrY, the detection of transcription of the *phrX* and *phrY* loci by RT-PCR, and the transcription of loci 72/101 and 102/16, and two supplementary tables showing the DNA oligonucleotides used in this study, and the matching sRNAs/ncRNAs detected by RNomics, Livny *et al.* (2006) and González *et al.* (2008), are available with the online version of this paper.

1995; Robertson & Roop, 1999). A *P. aeruginosa* PAO1*hfq*− mutant showed a reduced virulence in larvae of *Galleria mellonella* and in mice (Sonnleitner *et al.*, 2003). As in *E. coli* (Sledjeski *et al.*, 2001), Hfq is involved in regulation of *rpoS* expression in PAO1 (Sonnleitner *et al.*, 2003). However, Hfq also exerts σ^S-independent effects on catalase, pyocyanin and elastase production, and is required for PAO1 to swarm and twitch, which are important traits for colonization and biofilm development (Sonnleitner *et al.*, 2003). A comparative transcriptome analysis of a PAO1 *rpoS*− and a *rpoS*−*hfq*− strain has indicated that Hfq affects approximately 5 % of the PAO1 transcripts (Sonnleitner *et al.*, 2006). Among these transcripts, 72 are known to be regulated by QS, which is a cell density-dependent regulatory mechanism, impacting on virulence gene expression (Van Delden & Iglewski, 1998). Hfq has been suggested to affect QS and QS-controlled genes in at least two ways: by a non-specific positive regulation of the QS repressor QscR and of the *pqsH* gene, and by RsmY-mediated indirect positive regulation of the QS regulator RhlI (Sonnleitner *et al.*, 2006).

To date, the functions of only a small number of sRNAs are known in PAO1. The sRNAs RsmY (Valverde *et al.*, 2003) and RsmZ (Heurlier *et al.*, 2004) act by sequestration of the regulatory protein RsmA (Pessi *et al.*, 2001). As RsmA acts as a translational repressor of several virulence genes (Pessi *et al.*, 2001), RsmY and RsmZ RNA stimulate indirectly the synthesis of these virulence factors. The sRNAs PrrF1 and PrrF2 (Wilderman *et al.*, 2004) are orthologues of the *E. coli* sRNA RyhB, and are involved in regulation of iron acquisition and storage functions.

While this work was in progress, 25 small PAO1 RNAs of unknown function have been computationally predicted based on sequence conservation, and their expression has been experimentally verified (Livny *et al.*, 2006; González *et al.*, 2008). In contrast to those studies, here we have used (i) a shotgun-cloning approach (RNomics) (Vogel *et al.*, 2003), and (ii) bioinformatics tools based on the evolutionary conservation of RNA structure rather than on sequence conservation to reveal candidate sRNAs in PAO1. In summary, RNomics revealed three novel sRNA candidates, PhrD, PhrX and PhrY, the transcriptional start sites of which were analysed. Another sRNA, PhrS, which has likewise been identified in recent screens (Livny *et al.*, 2006; González *et al.*, 2008), was studied in more detail. The bioinformatic tool RNAz (Washietl *et al.*, 2005a) identified seven novel sRNA and/or ncRNA loci, of which two were shown to be transcribed. A compilation of matching PAO1 sRNAs/ncRNAs detected by RNomics, RNAz, Livny *et al.* (2006) and González *et al.* (2008) is provided.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. The bacterial cultures were grown at 37 °C in Luria–Bertani (LB) medium (Miller, 1972) supplemented with appropriate antibiotics. Antibiotics were added to final concentrations of 200 μg spectinomycin ml^{-1}, 200 μg streptomycin ml^{-1}, 50 μg gentamicin ml^{-1}, 125 μg tetracycline ml^{-1}, 100 μg ampicillin ml^{-1} and 100 μg rifampicin ml^{-1}.

RNomics. Total RNA was extracted from PAO1 cultures grown to early stationary phase (LB medium; OD_{600}=2) and from PAO1 cultures, which were grown in 200 ml LB to OD_{600} 1.0, followed by further incubation for 4 h in 50 ml PBS buffer (160 mM NaCl, 4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 15 % non-inactivated human serum HS-AB-100 (PromoCell). The cells were resuspended in VD buffer (10 mM Tris/HCl, pH 7.4, 6 mM NH_4Cl, 6 mM β-mercaptoethanol) containing 2 mM magnesium acetate, and lysed by sonication. The extracts were centrifuged at 150 000 ***g*** for 6 h to pellet ribosomes and rRNA. The supernatant was treated with DNase I (Fermentas), and the RNA was purified by phenol/chloroform extraction. Total RNA was size-fractionated on denaturing 6 % polyacrylamide/8 M urea gels. RNAs in the range

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype/relevant features	Source/reference
***P. aeruginosa* strains**		
PAO1	Wild-type	Holloway *et al.* (1979)
PAO1*hfq*−	PAO1 *hfq*::*aadA*, Sm/Sp^R	Sonnleitner *et al.* (2003)
***E. coli* strains**		
Top10		Stratagene
Plasmids		
pGEM-T Vector		Promega
pME4510	Broad–host-range promoter-probe plasmid, Gm^R	Rist & Kertesz (1998)
pME*phrD*	pME4510 harbouring *phrD* (authentic promoter)	This study
pME*phrS*	pME4510 harbouring *phrS* (authentic promoter)	This study
pUC19	*colE1* ori, Amp^R	Yanisch-Perron *et al.* (1985)
pUC19-T7*phrD*	pUC19 bearing *phrD* under T7 promoter control	This study
pUC19-T7*phrS*	pUC19 bearing *phrS* under T7 promoter control	This study
pME6013/14/15	Cloning vectors for translational *lacZ* fusions, Tc^R	Schnider-Keel *et al.* (2000)
pME9651	pME6013 with *phrs*-ORF′–′*lacZ*	This study
pME9651-1	pME6013 with *phrs*-$ORF_{AUG\rightarrow CUG}$′–′*lacZ*	This study

between ~50 and 500 nt were excised from the gel, eluted and ethanol-precipitated. Ten micrograms of gel-purified RNA was added to 1 nmol purified PAO1 Hfq. After 10 min on ice, 10 µl polyclonal rabbit anti-Hfq IgG was added and incubation was extended for 45 min on ice. Then, 20 µl Dynabeads Protein G (Dynal Biotech), washed once with 0.1 M sodium acetate and twice with VD buffer containing 2 mM magnesium acetate, was added. After 40 min at room temperature the mixture was subjected to a magnetic device (Dynal MPC) to capture Hfq-bound RNAs. To remove unbound components, the beads were washed four times with VD buffer containing 2 mM magnesium acetate and 480 mM NH_4Cl. The Hfq-bound RNAs were eluted from the beads by phenol/chloroform extraction, and subsequently precipitated with ethanol. For the cDNA library, the RNAs were polyC-tailed by poly(A) polymerase (Invitrogen), the C-tailed RNAs were ligated to a 5′ oligonucleotide linker, and the RNAs were then converted into cDNA by RT-PCR, as described by Hüttenhofer & Vogel (2006), employing primers complementary to the 5′ linker and the poly(C) tail, followed by cloning into the pGEM-T vector (Promega). The cDNA clones were sequenced (Lung *et al.*, 2006), and the cDNA sequences were compared with one another using the Lasergene Seqman II program package to identify identical sequences (DNASTAR). Following a BLASTN search against the PAO1 genome (http://www.pseudomonas.com), all sequences corresponding to intergenic regions or to the opposite strand of protein-coding regions were considered to encode potential novel sRNAs/ncRNAs.

Construction of plasmids and RNA preparation. For *in vitro* transcription of PhrD and PhrS, the plasmids pUC19-T7*phrD* and pUC19-T7*phrS* (Table 1) were constructed by inserting the PCR products generated with primer pair Z32 and Y32 (*phrD*; Supplementary Table S1), and primer pair J33 and I33 (*phrS*; Supplementary Table S1), respectively, into the *Xba*I–*Pst*I site of plasmid pUC19. The forward primers Z32 and J33 contained a T7Φ10 promoter sequence.

The plasmids pME9651 and pME9651-1, harbouring the *phrS*-ORF′–′*lacZ* and the *phrS*-$ORF_{AUG\rightarrow CUG}$′–′*lacZ* fusion genes, respectively, were constructed as follows. The promoter region of *phrS* and the first 47 bp of *phrS* were amplified (Fig. 1) using primer C2_phrSlacZfw together with primer D2_phrSlacZrev or primer O5_phrSstop (Supplementary Table S1) and genomic DNA of PAO1 as template. The PCR fragments were digested with *Eco*RI and *Pst*I and ligated into the corresponding sites of pME6013 (Table 1), resulting in pME9651 and pME9651-1, respectively.

For overproduction of the PhrD and PhrS RNAs, the plasmids pME*phrD* and pME*phrS* (Table 1) were constructed. The respective DNA sequences of *phrD* and *phrS*, including their authentic promoters, were amplified by PCR using the primer pairs T33 and Y32 for *phrD* and H33 and I33 for *phrS* (Supplementary Table S1), containing a *Bam*HI or *Pst*I restriction site (highlighted in bold type in Supplementary Table S1). The PCR fragments were then cloned into the corresponding sites of plasmid pME4510 (Table 1), resulting in pME*phrD* and pME*phrS*, respectively.

Northern blotting. Total RNA of PAO1 and PAO1*hfq*− was purified using the hot phenol method (Lin-Chao & Bremer, 1986). The abundance of the PhrD and PhrS RNAs was determined by Northern blotting using 15 µg total RNA. The signals were normalized to the hybridization signal obtained for ribosomal 5S rRNA, as described by Sonnleitner *et al.* (2006). The RNA samples were denatured for 5 min at 65 °C in loading buffer containing 50 % formamide, separated on 8 % polyacrylamide/8 M urea gels, and then transferred to nylon membranes by electroblotting. The RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized with gene-specific ^{32}P-end-labelled oligonucleotides (PhrD, P31; PhrS, O30; 5S rRNA, I26; see Supplementary Table S1), and the hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

Determination of the half-lives of PhrD and PhrS in PAO1 and PAO1*hfq*−. PAO1 and PAO*hfq*− were grown in LB medium to OD_{600} 2.0, then rifampicin (final concentration 100 µg ml^{-1}) was added to both strains and 10 ml aliquots were withdrawn at 0, 5, 15, 30 and 45 min thereafter for isolation of total RNA (see above). Total RNA (6 µg) of each sample was loaded on an 8 % polyacrylamide/8 M urea gel and blotted to a Hybond-N membrane (Amersham). PhrD and PhrS were visualized with DIG-labelled double-stranded probes. Chromosomal DNA of PAO1 was used as a template for the DIG-labelling procedure, as described by the manufacturer (DIG DNA Labeling Mix, Roche) with the primer pairs PhrDfw and PhrDrev for PhrD, PhrSfw and PhrSrev for PhrS, and 5S-rRNA-1 and 5S-rRNA-2 for 5S rRNA (Supplementary Table S1), which served as a loading control.

Primer extension analysis. For determination of the 5′ ends of PhrD and PhrS (Fig. 2), and PhrX and PhrY (Supplementary Fig. S1), total RNA was purified using the hot phenol method (Lin-Chao & Bremer, 1986) after growth of PAO1 in LB medium to OD_{600} 2.0. Primer extension was performed with AMV reverse transcriptase (Promega) using 2 µg total RNA and the 5′ end-labelled oligonucleotides P31 for PhrD, O30 for PhrS, I37 and B32 for PhrX, and K37 and R30 for PhrY (Supplementary Table S1). The plasmids pME*phrD* and pME*phrS* were used as templates in DNA sequencing reactions.

To verify overexpression of the plasmid-encoded PhrS RNA, total RNA was purified from PAO1 harbouring plasmids pME4510 or pME*phrS* in LB medium during exponential growth (OD_{600}=0.8), or upon growth to early stationary (OD_{600}=1.5) or stationary phase (OD_{600}=2.5). The primer extension experiments were carried out as mentioned above, and the normalization of the primer extension signal for PhrS to the 5S rRNA signal was performed as described by Sonnleitner *et al.* (2006).

RT-PCR. Total RNA of PAO1 was purified using the RNA/DNA Maxi kit for low-molecular-weight RNAs (Qiagen). Purified RNA (10 µg) was treated with 20 U RNase-free DNase I (Roche). For cDNA synthesis, 20 pmol of primers I37 (PhrX), K37 (PhrY), T34 (RsmY), E47 (RNA 72/101) and G47 (RNA 102/16) (Supplementary Table S1) were annealed to 2 µg RNA for 2 min at 80 °C. Upon cooling on ice, RNase-free AMV Reverse Transcriptase buffer (Promega) and dNTPs (10 mM) in a total volume of 20 µl were added. Then, 30 U AMV reverse transcriptase (Promega) was added, and the reaction was allowed to proceed for 1 h at 42 °C. Aliquots of 0.5 µl of these cDNA reactions were used as templates in 25 µl PCR amplification reactions using primers H37 and I37 for PhrX, J37 and K37 for PhrY, G40 and T34 for RsmY, D47 and E47 for RNA 72/101, and F47 and G47 for RNA 102/16 (1 µM final concentrations; Supplementary Table S1) and GoTaq Green Master Mix (Promega). The PCR fragments generated were analysed on 6 % polyacrylamide gels stained with ethidium bromide. Chromosomal DNA of PAO1 was used as a positive control and RT-PCR performed in the absence of reverse transcriptase was used as a negative control.

Gel mobility shift assays. The PhrD and PhrS RNAs were transcribed *in vitro* using T7 polymerase (Fermentas) and the *Pst*I-linearized plasmids pUC-T7*phrD* and pUC-T7*phrS*, as described by Sonnleitner *et al.* (2006). The *in vitro*-transcribed RNAs were 5′ end-labelled with [γ-^{32}P]ATP (Amersham) and purified on 6 % polyacrylamide/8 M urea gels. Labelled RNA (0.05 pmol) was incubated with increasing amounts of purified Hfq hexamer protein (Hfq_6), as described by Sonnleitner *et al.* (2006). Non-labelled PhrD, PhrS and RsmY RNA, respectively, were used as specific competitors, and *E. coli* bulk tRNA as a non-specific competitor. Immediately before loading,

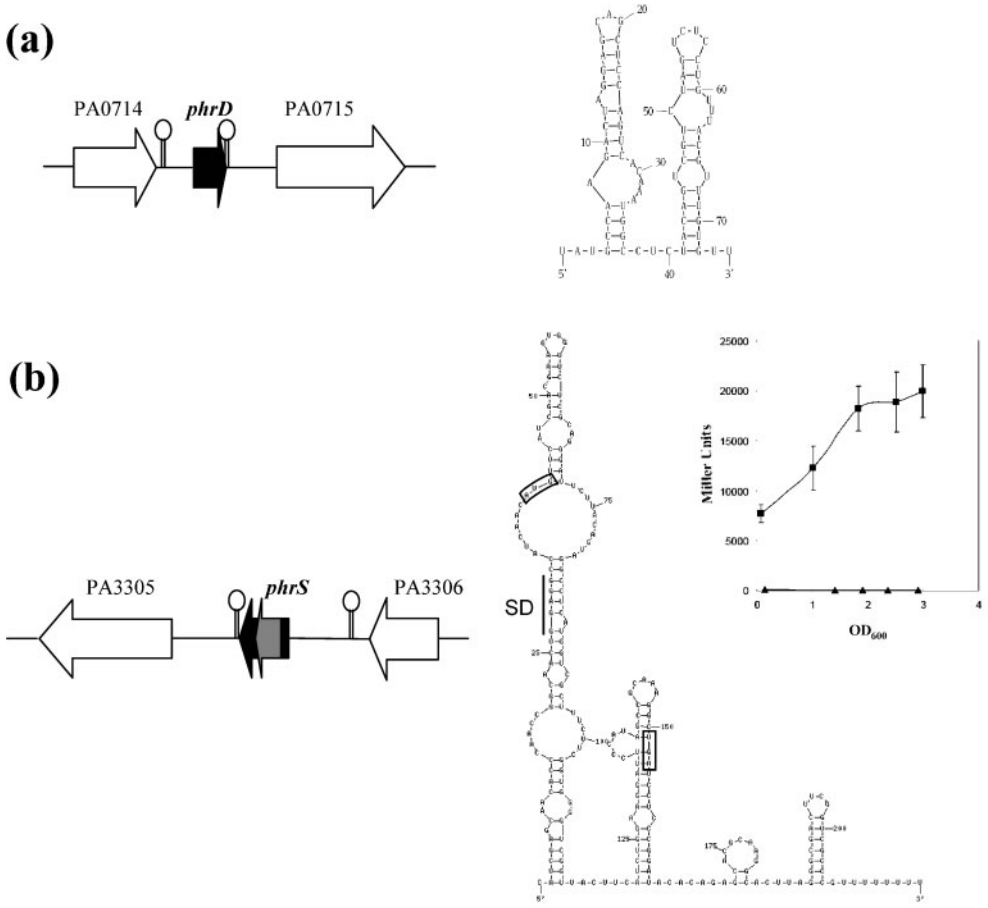


Fig. 1. Genetic organization and predicted secondary structures of PhrD and PhrS. (a) PhrD (black arrow) is located between the genes PA0714 and PA0715 (white arrows). (b) PhrS (black arrow) is located between the genes PA3305 and PA3306 (white arrows). Secondary structures of PhrD and PhrS predicted with mfold (Mathews *et al.*, 1999; Zuker, 2003) are shown on the right. The start and stop codons of the internal ORF encoded by PhrS are boxed. The putative Shine–Dalgarno (SD) sequence is indicated by a bar. Putative transcriptional terminators are indicated by stem–loop structures. Inset: expression of the *phrS*-ORF as evidenced by synthesis of the PhrSΦLacZ fusion protein. The β-galactosidase activities obtained with the strains PAO1(pME9651) (*phrS*-ORF–*lacZ*) (■) and PAO1(pME9651-1) (*phrS*-ORF$_{AUG\rightarrow CUG}$–*lacZ*) (▲) were determined as described by Miller (1972) at different growth phases. The experiment was performed in triplicate. Error bars, SD.

the samples were mixed with 4 µl loading dye (25 % glycerol, 0.2 mg xylencyanol l^{-1} and bromphenol blue), and loaded on a native 4 % polyacrylamide gel. Electrophoresis was performed in Tris-acetate/EDTA buffer at 160 V. The radioactively labelled bands were visualized with a PhosphorImager (Molecular Dynamics).

2D gel analysis. To detect PhrS-mediated regulatory effects, differences in the proteome profile were analysed, upon plasmid-directed overexpression of PhrS RNA. Total cellular extracts of strain PAO1 harbouring plasmid pME4510 or pME*phrS* were extracted and compared by 2D gel electrophoresis. The experiment was performed twice to ensure reproducibility. The strains were grown in LB medium to OD_{600} 1.5, and equal amounts of cells were dissolved in lysis buffer [8 M urea, 4 % (w/v) CHAPS, 40 mM Tris base]. The cells were disrupted by repeated freezing in liquid N_2 and thawing at 37 °C, followed by incubation at 37 °C for 1 h to achieve complete lysis. For the first dimension, the Immobiline Dry Strip pH 3–10 (18 cm; Amersham Pharmacia Biotech) was used with the following IEF program: 12 h rehydration at 40 V, 0.5 h at 300 V, 0.5 h at 1000 V, 0.5 h at 2000 V, 0.5 h at 3000 V, 9 h at 7000 V at 18 °C (IPGphor isoelectric focusing system). Resolution in the second dimension was performed on 12 % SDS-polyacryamide gels for 15 min at 15 mA, and then for 4 h at 20 mA. Buffers and conditions were used according to the manufacturer's instructions. The gels were silver-stained as described elsewhere (Shevchenko *et al.*, 1996). Selected protein spots were excised from the gel and the protein identities were assessed by MS.

RNAz screen. The tool NcDNAlign (Rose *et al.*, 2008) was used to construct multiple sequence alignments based on the known genome sequences of *P. aeruginosa* PAO1 (NC 002516), *P. aeruginosa* UCBPP-PA14 (NC 008463), *Pseudomonas entomophila* (NC 008027), *Pseudomonas fluorescens* Pf-5 (NC 004129), *P. fluorescens* PfO-1 (NC 007492), *Pseudomonas putida* KT2440 (NC 002947), *Pseudomonas syringae* pv. *phaseolicola* 1448A (NC 005773), *P. syringae* (NC 007005), *P. syringae* pv. tomato str. DC3000 (NC 004578) and *P. fluorescens* SBW25 (ftp://ftp.sanger.ac.uk/pub/pathogens/pf/PF.dbs). Briefly, the program is based on pairwise BLASTN (Altschul *et al.*, 1990)

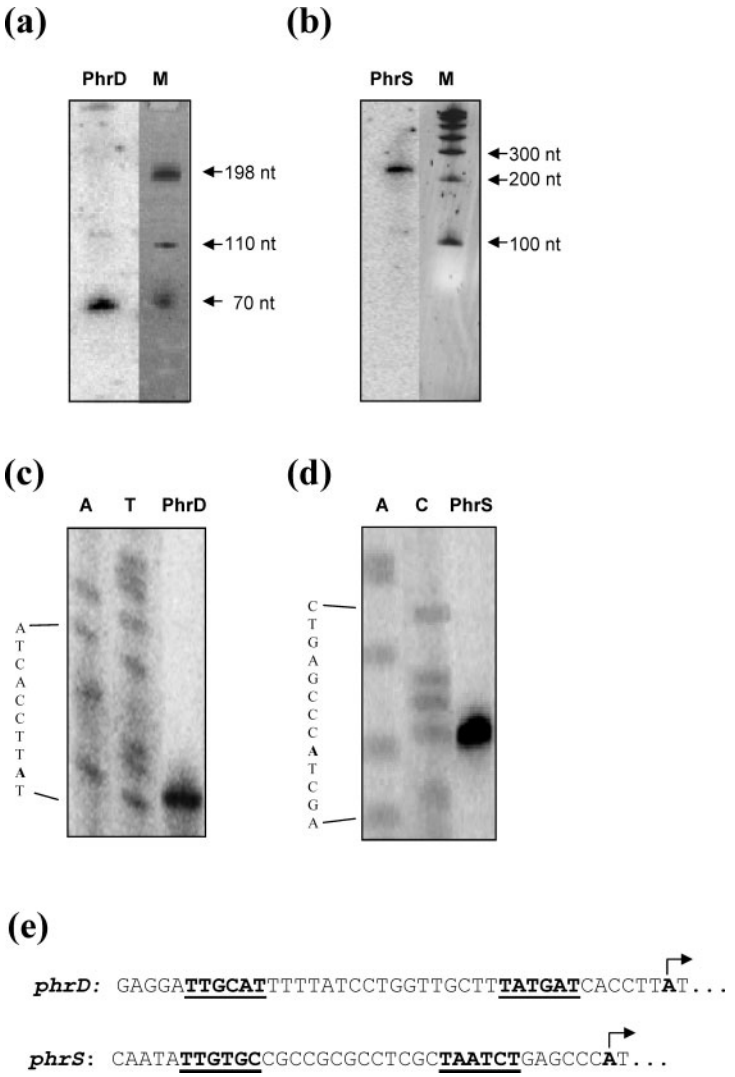


Fig. 2. Detection of PhrD (a) and PhrS (b) by Northern blot analyses. For the detection of PhrD, PAO1 cells were exposed to human serum as described in Methods. For the detection of PhrS, PAO1 was grown in LB medium to early stationary phase. Northern blots were performed with total RNA using strand-specific probes for PhrD and PhrS, as described in Methods. The size of RNA markers (M) is indicated on the right. Primer extension analyses were performed to determine the 5′ ends of PhrD (c) and PhrS (d) RNA. Lanes A, T and C represent sequencing ladders. The 5′ ends are indicated in bold type in the sequence. (e) Promoter sequences of *phrD* and *phrS*. The putative −10 and −35 boxes are in underlined bold type and the transcriptional start sites determined (c, d) are highlighted in bold type and marked with an arrow.

(e-value $<10^{-3}$) comparisons, which are combined to multiple alignments. For comparison, MultiZ (Blanchette *et al.*, 2004) was additionally used to produce an alternative set of genome-wide alignments. Both alignments were used as input for the RNAz pipeline (Washietl *et al.*, 2005a, b). The RNAz approach searches for signatures of conservation of RNA secondary structure in a multiple sequence alignment and uses a support vector machine (SVM) to distinguish conserved RNA structure elements from genomic background. In order to assess the reliability of the predictions, the procedure was repeated two times with two different sets of input alignments. In each case the procedure outlined in the current version of the RNAz manual was used (Washietl, 2006). As the SVM of RNAz cannot handle more than six aligned sequences, from alignments exceeding six, the pipeline selects a subset of six sequences that have approximately equal pairwise sequence similarities. Input alignments are cut into windows of length 120 with 40 nt overlap between adjacent slices, which are scored individually. Finally, the predictions are combined to contiguous structured loci at the reference genome, in this case PAO1. As RNAz returns a classification confidence, we report the results for two cutoff values: $p > 0.5$ and a high confidence set with $p > 0.9$. The predicted structured RNAs were compared using BLASTN to the available public databases, the sequences reported by Livny *et al.* (2006) and González *et al.* (2008), and the Hfq-binding RNAs listed in Table 2. To identify known PAO1 ncRNAs among the predictions, rnazAnnotate.pl and a minimum overlap of 0.7 were used.

RESULTS AND DISCUSSION

Identification of ncRNAs and sRNA candidates by Rnomics

With the aim of identifying sRNAs in PAO1, we used an RNomics approach, which is based on the generation of specialized cDNA libraries encoding potential novel sRNA species (Hüttenhofer *et al.*, 2004; Hüttenhofer & Vogel, 2006; Vogel *et al.*, 2003). In an initial attempt, we shotgun-cloned size-fractionated total RNA (50–300 nt). However, as revealed by subsequent sequencing, this approach yielded mainly RNA fragments originating from rRNA and tRNA (not shown). In a second experiment, a cell extract was used for co-immunoprecipitation of Hfq-binding RNAs with PAO1-Hfq specific antibodies upon

Table 2. RNAs mapping to intergenic regions of PAO1

sRNA*	Adjacent genes	Strand†	Growth‡	5′ end§	3′ end‖	Length¶	Identified sequence#
RsmY	*dnr*/PA0528	← →←	S	586 867	586 990	124	586 867–586 960
PhrC	PA0667/*tyrZ*	← →→	LB				720 082–720 136
PhrD^{NB}	PA0714/PA0715	→→→	S/LB	785 497	785 570	72	785 498–785 547
tmRNA	PA0826/PA0827	←←←	LB				901 536–901 640
PhrR	*mobA*/PA3031	→← →	LB				3 394 727–3 394 805
PhrS(P20/1887)**	PA3305/PA3306	←←←	LB	3 705 522	3 705 309	212	3 705 342–3 705 515
amiE leader	*amiE*/PA3367	←← →	LB	3 778 134	3 778 034	100	3 778 054–3 778 098
PhrU	PA3868/PA3869	→← →	S				4 332 627−4 332 676
PhrW(P2/2510)**	PA4421/PA4422	←←←	LB				4 956 348–4 956 591
PhrX$^{RT\text{-}PCR}$	PA5183/PA5184	→← →	S	5 836 429	5 836 579	151	5 836 450–5 836 479
PhrY$^{RT\text{-}PCR}$	*argA*/PA5205	← →→	LB	5 859 480	5 859 674	195	5 859 471–5 859 616

*New sRNA candidates revealed by RNomics detected by Northern-blotting $^{(NB)}$ or by RT-PCR $^{(RT\text{-}PCR)}$ are highlighted in bold type.
†The middle arrow indicates the orientation of the RNAs, while the flanking arrows indicate the orientation of the adjacent genes. Genes present on the strand given in the PAO1 genome database (http://v2.pseudomonas.com) (→); genes present on the complementary strand (←).
‡sRNA fragments were detected in PAO1 grown to early stationary phase in LB medium (LB) and/or after serum exposure (S) as described in Methods.
§Determined by primer extension.
‖Calculated from Northern blot experiments and the presence of a putative rho-independent terminator.
¶The calculated RNA length includes the U-run.
#Identified sequence by RNomics, the numbers represent the location on the PAO1 genome (http://v2.pseudomonas.com).
**PhrS and PhrW were recently detected as P20/1887 and P28/2510, respectively (Livny *et al.*, 2006; González *et al.*, 2008). PhrW/P28/2510 likely corresponds to RnpB, which in *E. coli* is a component of RNase P (Livny *et al.*, 2006).

removal of ribosomes by centrifugation. Although this modification of the protocol significantly decreased the number of rRNA clones, the majority of the cloned RNAs originated from larger mRNA fragments, in accordance with the capacity of Hfq to bind to mRNAs (Vecerek *et al.*, 2003). Therefore, total RNA obtained after removal of ribosomes was first size-fractionated (50–300 nt), followed by co-immunoprecipitation of Hfq-binding RNAs. The procedure was carried out with cells grown to early stationary phase or with cells exposed to human serum (see Methods) to mimic systemic PAO1 infection (Van Delden, 2004). The protocol used here deviates from the co-immunoprecipitation of sRNA with Hfq antibodies from cell lysates (Zhang *et al.*, 2003), and is biased against sRNAs that eventually associate with ribosomes (Worhunsky *et al.*, 2003).

A total of 400 cDNA sequences were analysed by first grouping identical cDNA clones, followed by their location by bioinformatics on the PAO1 genome. Although the cell extracts were centrifuged at 150 000 ***g*** to remove ribosomes, ~15 % of the sequenced clones from stationary-phase or serum-treated cells represented rRNA fragments. Of the clones, 40 and 20 % were derived from coding regions of PAO1 (presumably representing mRNA degradation intermediates) and from the opposite strand of protein-coding regions, respectively. Eleven candidate sRNA-encoding genes (Table 2) were predicted to localize to intergenic regions based on the presence of putative rho-independent terminators.

Of the 11 candidate sRNAs, two ncRNAs, RsmY and tmRNA (Table 2), have been previously described (Sonnleitner *et al.*, 2006; Williams & Bartel, 1996). When PAO1 was exposed to human serum, the RsmY RNA gene was repeatedly present in the cDNA library (nine cDNA clones were found), which verified the RsmY interaction with Hfq (Sonnleitner *et al.*, 2006) and could point to its involvement in the regulation of virulence factors (Kay *et al.*, 2006). The tmRNA, also known as *ssrA* RNA or 10Sa RNA, functions as both tRNA and mRNA (Komine *et al.*, 1994). A fragment of the PAO1 tmRNA (Table 2) co-immunoprecipitated with Hfq. However, as the *E. coli* tmRNA does not bind to Hfq (Wassarman *et al.*, 2001) and the PAO1 tmRNA has previously been annotated, we did not further study whether full-length PAO1 tmRNA binds to Hfq. In addition, one Hfq-binding RNA fragment was identified as part of the leader RNA of the amidase operon (Table 2; Drew, 1984; Wilson & Drew, 1995), and another Hfq-binding RNA termed PhrW (Table 2) corresponded to P28/2510, which was recently detected by Livny *et al.* (2006) and González *et al.* (2008). PhrW/P28/2510 shows significant homology with RnpB, which in *E. coli* is part of RNase P (Livny *et al.*, 2006). Because of their known or inferred function, the last two RNAs were likewise not further studied.

Next, specific probes were designed for the detection of the remaining seven sRNA candidates by Northern hybridization performed with total RNA from PAO1 cultures grown to early stationary phase and after exposure to human

serum. Only two *Pseudomonas* Hfq-binding RNAs, termed PhrD and PhrS (Fig. 1a, b), isolated from cells exposed to serum and from stationary-phase cells, respectively, were detected (Fig. 2a, b, Table 2). PhrS matched with the recently described, but not further characterized, sRNA P20/1887 (Livny *et al.*, 2006; González *et al.*, 2008). From the determination of their major 5′ ends by primer extension (Fig. 2c, d), and from the position of the putative transcriptional terminator, the sizes of these sRNAs were estimated to be 72 nt (PhrD) and 212 nt (PhrS), which was in agreement with the size of the corresponding signals detected on Northern blots (Fig. 2a, b). A putative −10 box (TATGAT) and a putative −35 box (TTGCAT) were found upstream of the predicted transcription start site of *phrD*. A putative σ^{70} promoter with a −10 box (TAATCT) and a −35 box (TTGTGC) was likewise detected upstream of the transcriptional start site of *phrS* (Fig. 2e). However, with only 13 bp, the spacing between the −35 and the −10 region would deviate from that of canonical σ^{70} promoters. Nonetheless, as shown below (Fig. 5), this putative promoter was able to direct transcription of the plasmid-borne *phrS* gene. A BLASTN search of PhrD with all bacterial sequences in the NCBI homepage (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) revealed that PhrD is only present in PAO1, whereas homologues of PhrS are found in different isolates of *P. aeruginosa* (PACS2, C3719, 2192, UCBPP-PA14 and PA7), although not in other Gram-negative bacteria.

Among the remaining five candidate sRNA genes, the transcription of *phrX* and *phrY* sequences could be verified by RT-PCR (Supplementary Figs S1 and S2a, b). PhrX and PhrY were isolated from cDNA libraries generated from cells exposed to human serum and from cells grown to early stationary phase, respectively. From the primer extension analyses (Supplementary Fig. S2c), and from the position of the putative transcriptional terminator, the sizes of these RNAs were calculated to be 151 nt (PhrX; Supplementary Fig. S1) and 174 nt (PhrY; Supplementary Fig. S1), respectively. PhrX and PhrY are transcribed from the intergenic regions between PA5183 and PA5184 (Supplementary Fig. S1a, Table 2) and between *argA*, encoding an *N*-acetylglutamate synthase, and PA5205, encoding a conserved hypothetical membrane protein (Supplementary Fig. S1b, Table 2), respectively.

PhrS contains an ORF

Inspection of the *phrS* gene revealed an ORF with the capacity to encode a 37 aa peptide (Fig. 1b). To date, only a few regulatory RNAs have been shown to have protein-coding capacity. For instance, the RNAIII gene of *Staph. aureus* contains an ORF *hld*, which encodes delta-haemolysin (Janzon & Arvidson, 1990), and the *E. coli* SrgS sRNA, besides being a riboregulator, encodes the 43 aa SgrT polypeptide with a function in glucose uptake (Wadler & Vanderpool, 2007). To test whether the internal ORF of *phrS* (*phrS*-ORF) is expressed, a translational fusion between the *phrS*-ORF and the *lacZ* reporter gene was engineered. In plasmid pME9651 transcription of the *phrS*-ORF–*lacZ* gene is driven by the authentic *phrS* promoter. The control plasmid pME9651-1, which harbours the same *phrS*-ORF–*lacZ* gene, but wherein the start codon of *phrS*-ORF is changed to a CUG ($phrS\text{-}ORF_{AUG\rightarrow CUG}\text{–}lacZ$), did not direct synthesis of the fusion protein. In contrast, plasmid pME9651 directed synthesis of the PhrSΦLacZ protein (Fig. 1b, inset), indicating that the internal ORF of *phrS* is indeed translated. Thus, PhrS appears to be another candidate for a bifunctional sRNA acting as a riboregulator (see below) and as mRNA. The function of the encoded peptide remains to be elucidated.

Binding of PhrD and PhrS to Hfq

As PhrD and PhrS were abundant RNA species (Fig. 2), we focused in further experiments on these sRNAs. First, we verified that they bind to Hfq by performing band-shift assays with purified PAO1 Hfq protein. Hfq_6 was added in increasing molar ratios to 5 nM of the respective 5′ end-labelled RNA. When Hfq_6 was added to PhrD in a molar ratio of 1 : 1, two band shifts were observed (Fig. 3a, lane 2; B1 and B2). However, the amount of PhrD present in shift B2 was only marginal and did not increase with higher concentrations of Hfq_6. The apparent K_d value was 5.5 ± 3.7 nM when 50 % of PhrD RNA was present in complex B1. The competition experiment suggested that PhrD binds specifically to Hfq. Unlabelled PhrD RNA competed with the Hfq–PhrD complex (Fig. 3a, lane7) when added in twofold molar excess over Hfq, whereas the non-specific competitor *E. coli* tRNA did not (Fig. 3a, lanes 8–9).

When Hfq_6 was added in increasing molar excess to PhrS RNA, two bands were observed (Fig. 3b, lanes 2–5; B1 and B2). The second shift could indicate a 2 : 1 stoichiometry of Hfq_6 to PhrS, which is consistent with the observation that the second shift is more prominent at a higher molar excess of Hfq_6 (Fig. 3b, lane 5). The apparent K_d values were 13.9 ± 3.6 nM for the first shift and 32.2 ± 6.8 nM for the supershift. Binding of Hfq to PhrS RNA was verified as described above with unlabelled PhrS RNA (Fig. 3b, lanes 6 and 7), with the Hfq-binding PAO1 RsmY RNA (Fig. 3b, lanes 8 and 9) (Sonnleitner *et al.*, 2006), and the non-specific competitor *E. coli* tRNA (Fig. 3b, lanes 10 and 11).

Abundance of PhrD and PhrS in the presence and absence of Hfq

To test whether Hfq affects the abundance of PhrD and PhrS, their steady-state levels were assessed by Northern blotting using total RNA isolated from PAO1, from PAO1*hfq*− grown in LB medium to OD_{600} 2 (Fig. 4), or after growth in LB to OD_{600} 1.0, followed by exposure to non-inactivated human serum in PBS buffer (Serum; Fig. 4). When compared with PAO1, the steady-state level of PhrD was reduced ~50 % in the *hfq*− strain under both

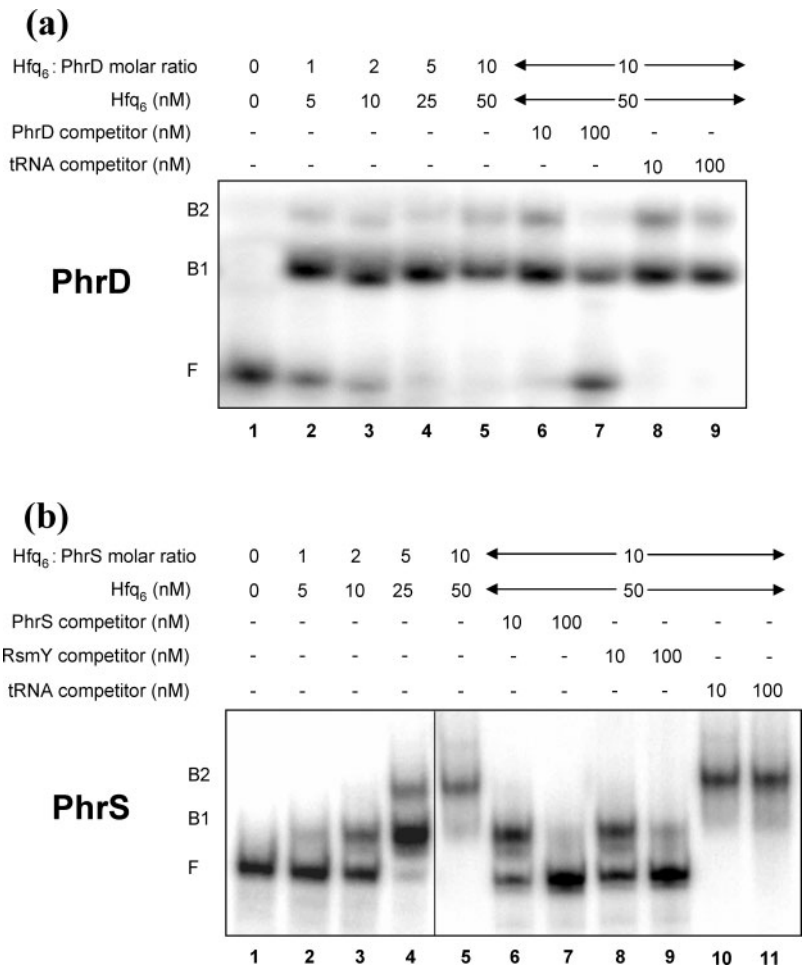


Fig. 3. Hfq binds to PhrD (a) and PhrS (b). Labelled RNA (5 nM) was incubated in the absence (lane 1) or in the presence of 5 nM (lane 2), 10 nM (lane 3), 25 nM (lane 4) and 50 nM (lane 5) Hfq_6. The Hfq_6 : RNA ratio is indicated at the top. Unlabelled PhrD and PhrS RNA (lanes 6 and 7), PAO1 RsmY RNA (b, lanes 8 and 9) and tRNA (a, lanes 8 and 9; b, lanes 10 and 11) were added as competitors. The positions of free (F) and Hfq-bound (B) RNA are indicated.

conditions (Fig. 4a). PhrS was predominantly synthesized in early stationary phase, and was absent upon serum exposure (Fig. 4b). When compared to PAO1, the steady-state level of PhrS was ~50 % reduced in the PAO1*hfq*− mutant (Fig. 4b). These experiments indicated that Hfq affects the abundance of PhrD and PhrS, which could result from direct or indirect effects of Hfq on transcription of the two sRNAs, or from Hfq-mediated protection of these sRNAs from degradation (Moll *et al.*, 2003a; Sorger-Domenigg *et al.*, 2007). To distinguish between these possibilities, we next determined the half-lives of both sRNAs. The half-lives of PhrD and PhrS were comparable in PAO1 and in the PAO1*hfq*− mutant strain (Fig. 4c). Hence, Hfq appears to affect expression of these sRNAs rather than stabilizing them.

Expression of chromosomally and plasmid-encoded *phrS* at different growth phases

Next, we tested whether expression of *phrD* and *phrS* from plasmids pME*phrD* and pME*phrS*, respectively, resulted in an enhanced level of the corresponding RNAs. This was done with the aim of using plasmid-mediated over-production of the respective RNAs for the identification of possible target genes in proteome studies. Several efforts to transform plasmid pME*phrD* in PAO1 failed, which could suggest that overexpression of *phrD* is lethal. Therefore, these studies were only continued with PhrS. As PhrS was isolated from cells grown to stationary phase, we first determined the steady-state levels of PhrS in LB medium during exponential growth (OD_{600}=0.8) as well as in early (OD_{600}=1.5) and late stationary phase (OD_{600}=2.5). The plasmid- and chromosomally encoded PhrS RNA was predominantly expressed when cells entered stationary phase (Fig. 5a, lanes 3 and 4), whereas the abundance of PhrS was decreased at an OD_{600} of 2.5 (Fig. 5a, lanes 5 and 6).

Proteome analyses upon overproduction of PhrS

Under the premise that riboregulation by an sRNA can result in a decreased or increased synthesis of the protein encoded by the target mRNA, plasmid-directed over-expression of *phrS* followed by proteomics was used as a means to identify putative PhrS targets. When compared to chromosome-directed background synthesis in strain

Table 3. Summary of the NcDNAlign- and MultiZ-based RNAz screens

Shown are the counts of sRNA hits with a BLAST e-value cutoff of $1e^{-3}$ for the NcDNAlign and MultiZ approaches. The hits of each run are partitioned in two groups according to prediction confidence (p; >0.5 and 0.9, respectively). RNAz hits which map to existing annotations are given for sRNAs/ncRNAs listed in the PAO1 B GenBank (gpk), database entries in Rfam, Noncode, tmRDB and ncRNAdb, as well as for hits produced by tRNAscan.

sRNA hits	NcDNAlign		MultiZ	
	p ⩾0.5	p ⩾0.9	p ⩾0.5	p ⩾0.9
RNAz loci	115	98	221	166
RNAz windows	464	360	843	564
Annotated	101	89	85	74
Unknown	14	9	136	92
Overlap PAO1gbk-	88	78	60	55
Rfam	82	72	61	53
Noncode	5	3	6	5
tmRNA-db	1	0	1	1
ncRNA-db	5	4	4	2
tRNAscan	43	38	26	24
Livny *et al.* (2006)*	4	3	10	9
González *et al.* (2008)†	4	4	3	6
sRNAs RNomics‡	2	1	3	3

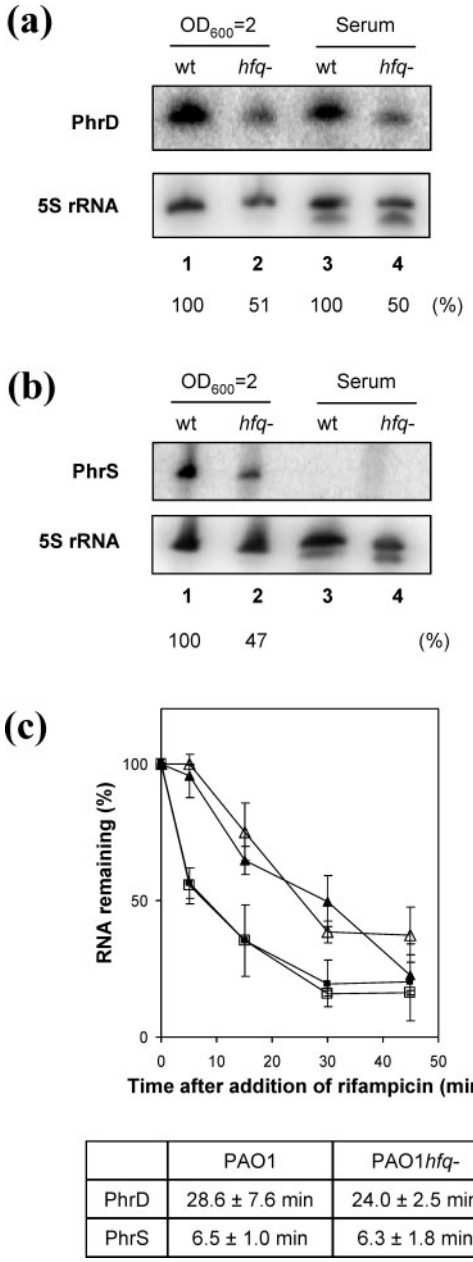
*RNAz hits that match with sRNAs/ncRNAs predicted or experimentally tested by Livny *et al.* (2006) are indicated.
†RNAz hits that match with sRNAs/ncRNAs predicted or experimentally tested by González *et al.* (2008) are indicated.
‡sRNAs identified by RNomics (see text).

PAO1(pME4510), the levels of PhrS were 12-fold increased in strain PAO1(pME*phrS*) when the cells entered early stationary phase (Fig. 5a, lanes 3 and 4). Therefore, samples for 2D gel analysis were withdrawn at OD_{600} 1.5. The 2D protein pattern of PAO1(pME4510) was compared to that of PAO1(pME*phrS*) (Fig. 5b), and three distinct protein spots were selected for identification by MS. The three possible targets of PhrS included the heat-shock chaperonin GroEL, the outer membrane porin OprD and the putative periplasmic binding protein PA5153, all of which were upregulated in the presence of increased levels of PhrS (Fig. 5b). GroEL is among the most highly conserved proteins in nature (Segal & Ron, 1996), and functions together with GroES to maintain protein integrity, which enables cells to survive a variety of environmental stresses (Hendrick & Hartl, 1993). A strong antibody response to GroEL has been found in cystic fibrosis patients with chronic pulmonary infection caused by *P. aeruginosa* (Ulanova *et al.*, 1997), which suggests a role for PhrS under these conditions. The outer membrane porin OprD has been shown to facilitate diffusion of basic amino acids as well as of small peptides, and also serves as a channel for the β-lactam antibiotic imipenem (Trias & Nikaido 1990), whereby the loss of OprD can lead to imipenem resistance (Lynch *et al.*, 1987). The proteome analysis upon overproduction of PhrS suggests that the sRNA could function as a riboregulator. Studies are under way to test whether PhrS regulates the identified protein genes in a direct or indirect manner.

PAO1 sRNAs predicted by RNAz

In addition to the RNomic approach, we made use of recently developed bioinformatics tools to search for sRNAs/ncRNAs in PAO1. The majority of the computational approaches to detect small ncRNAs in bacterial genomes, such as sRNAPredict (Livny *et al.*, 2006), search for putative genes without a recognizable ORF. In contrast, RNAz (Washietl *et al.*, 2005a) used here extracts information based on the evolutionary conservation of RNA structure from a multiple sequence alignment based on the notion that structured RNAs fold into more stable secondary structures than the genomic background sequence of the same composition. RNAz also evaluates the pattern of substitutions in a multiple sequence alignment of related species. Substitutions that are consistent with preserving a base pair (e.g. GC⟶GU) or that are compensatory (e.g. GC⟶UA) provide direct evidence for the conservation of secondary structure. RNAz uses an SVM approach to decide based on this input information whether a multiple sequence alignment contains a conserved RNA structure. As RNAz relies on both sequence and structure conservation across genomes, it cannot detect unstructured anti-sense regulators or species-specific sRNAs. Therefore, none of the candidate Hfq-binding sRNAs encoded in the opposite strand of protein-coding regions detected by RNomics was predicted by RNAz (not shown).

Using NcDNAlign alignments as input we found 115 structured candidate loci, of which 101 were previously



	PAO1	PAO1*hfq*-
PhrD	28.6 ± 7.6 min	24.0 ± 2.5 min
PhrS	6.5 ± 1.0 min	6.3 ± 1.8 min

Fig. 4. Steady-state levels of PhrD (a) and PhrS (b) in the presence (wt) or absence of Hfq (*hfq*–) at OD_{600} 2 upon growth in LB medium and after serum exposure (Serum; see text). (c) Stability of PhrD (triangles) and PhrS (squares) in the presence (PAO1; closed symbols) and absence of Hfq (PAO1*hfq*–; open symbols). Strains PAO1 and PAO1*hfq*– were grown in LB medium to OD_{600} 2.0. Total RNA was purified 0, 5, 15, 30 and 45 min after addition of rifampicin (100 μg ml^{-1} final concentration). The signals for the sRNAs were normalized to the respective signals of the 5S rRNA (loading control). The experiment was done in duplicate. Error bars, SD. The half-lives determined are given below the graph.

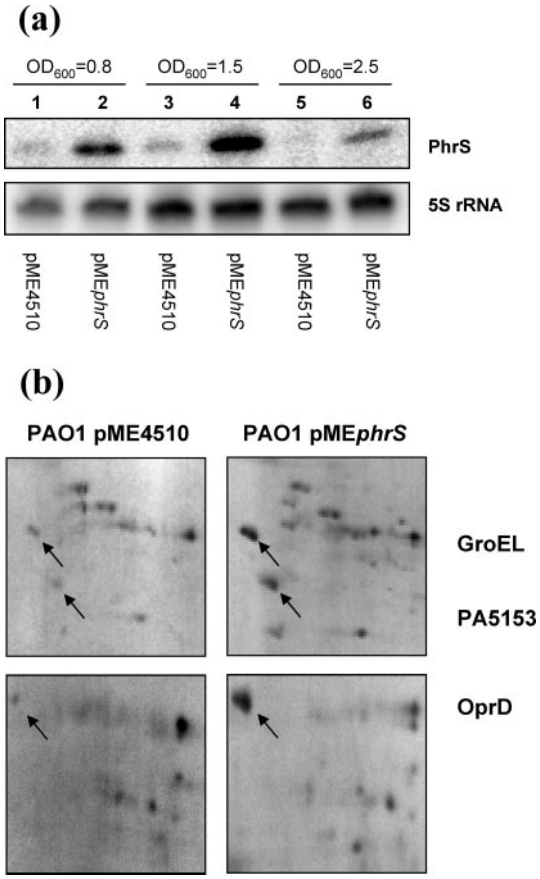


Fig. 5. (a) Comparison of the expression of plasmid-encoded *phrS* (a, lanes 2, 4 and 6; pME*phrS*) and chromosomally encoded *phrS* (a, lanes 1, 3 and 5; pME4510). Total RNA was extracted from cells during exponential growth (OD_{600}=0.8; lanes 1 and 2), upon growth to early stationary phase (OD_{600}=1.5; lanes 3 and 4) and to stationary phase (OD_{600}=2.5; lanes 5 and 6) in LB medium. In pME*phrS*, the *phrS* gene was cloned under the transcriptional control of its authentic promoter (see Fig. 1e). (b) Identification of putative targets of PhrS by proteomics. The protein pattern of PAO1(pME4510) (left-hand panels) was compared to that of PAO1 harbouring pME*phrS* (right-hand panels). The protein samples were taken in early stationary phase. Selected prominent spots of different intensity (black arrows) were identified by MS. Putative targets of PhrS were identified as GroEL, PA5153 and OprD. Only the relevant sections of the gels are shown.

known and 14 are novel sRNA/ncRNA predictions. Based on the less restrictive MultiZ alignments, 221 candidates were predicted, of which 85 correspond to known sRNAs/ncRNAs. Details of the predicted loci are provided at http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/. The results of the RNAz screen for both MultiZ- and NcDNAlign-generated alignments are shown in Table 3. The number of RNAz hits that matched with sRNAs /ncRNAs predicted or experimentally tested by Livny *et al.* (2006) and González *et al.* (2008) are indicated in Table 3. Information on these matches can be found at http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/. MultiZ-based alignments result in significantly more RNAz hits, both at high and low

Table 4. Novel RNAs predicted by both the MultiZ-based and the NcDNAlign-based runs

NcDNAlign run		MultiZ run	
Locus*	p†	Locus*	p†
1	0.88	41	0.93
4	0.81	44	0.83
52	0.99	75	0.99
72‡	0.94	101‡	0.94
96	0.99	167	0.98
102‡	0.99	16‡	0.96
115	0.50	99	0.95

*Information on the sRNAs/ncRNAs encoded by the different loci is available at http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/. The loci in each row encode the same putative sRNA/ncRNA.
†Prediction confidence; see Table 3.
‡Expression of these loci, i.e. RNAs, has been verified by RT-PCR.

confidence. NcDNAlign, however, identifies more annotatable hits than MultiZ at the cost of only a few novel sRNAs. Only 57 hits are shared between MultiZ- and NcDNAlign-based screens. Of these hits, only seven are novel, and all of them are high-confidence RNAz predictions (see Table 4). In an effort to demonstrate expression of these seven candidate sRNAs/ncRNAs, RT-PCR was exploited. As shown in Supplementary Fig. S3, transcription of the sRNAs/ncRNAs encoded by the loci 72/101 and 102/16 (Table 4) predicted by NcDNAlign and MultiZ, respectively, could be verified.

Three Hfq-binding sRNAs detected by RNomics were likewise predicted by the RNAz screens. PhrW (input NcDNAlign, locus 73; input MultiZ, locus 100; probable RNase P component; P28 in Livny *et al.*, 2006) and PhrH (input NcDNAlign, locus 20; input MultiZ, locus 77), which represents tmRNA, are highly conserved among pseudomonads (see http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/). PhrS, which corresponds to P20 in Livny *et al.* (2006) and 1887 in González *et al.* (2008), was also predicted by RNAz using MultiZ alignments as input (locus 114; see http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/). As PhrS emerged from four different screens (Livny *et al.*, 2006; González *et al.*, 2008; Tables 2 and 3) and *phrS* overexpression resulted in changes in the protein pattern, it appears to be a prime candidate for an sRNA in *Pseudomonas*. All other sRNA candidates detected by RNomics, which mapped to intergenic regions (Table 2), were not found in the RNAz screen, indicating that they are species-specific. Likewise, none of them was identified by Livny *et al.* (2006) and González *et al.* (2008). A compilation of matching sRNAs/ncRNAs predicted and/or identified by RNomics, RNAz, and by Livny *et al.* (2006) and González *et al.* (2008) is shown in Supplementary Table S2.

ACKNOWLEDGEMENTS

We thank Dr Dieter Haas for providing materials. This work was supported by grant F1715 from the Austrian Science Fund (U. B.), the GEN-AU project D1042-011-011 (A. H.) and by EU-FP6-grant 018618 (I. M.).

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E. G., Margalit, H. & Altuvia, S. (2001). Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol* **11**, 941–950.

Blanchette, M., Kent, W. J., Riemer, C., Elnitski, L., Smit, A. F., Roskin, K. M., Baertsch, R., Rosenbloom, K., Clawson, H. & other authors (2004). Aligning multiple genomic sequences with the threaded blockset aligner. *Genome Res* **14**, 708–715.

Ding, Y., Davis, B. M. & Waldor, M. K. (2004). Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression. *Mol Microbiol* **53**, 345–354.

Drew, R. (1984). Complementation analysis of the aliphatic amidase genes of *Pseudomonas aeruginosa*. *J Gen Microbiol* **130**, 3101–3111.

González, N., Heeb, S., Valverde, C., Kay, E., Reimmann, C., Junier, T. & Haas, D. (2008). Genome-wide search reveals a novel GacA-regulated small RNA in *Pseudomonas* species. *BMC Genomics* **9**, 167.

Gottesman, S. (2005). Micros for microbes: non-coding regulatory RNAs in bacteria. *Trends Genet* **21**, 399–404.

Guillier, M., Gottesman, S. & Storz, G. (2006). Modulating the outer membrane with small RNAs. *Genes Dev* **20**, 2338–2348.

Hendrick, J. P. & Hartl, F. U. (1993). Molecular chaperone functions of heat-shock proteins. *Annu Rev Biochem* **62**, 349–384.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P. & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186**, 2936–2945.

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

Hüttenhofer, A. & Vogel, J. (2006). Experimental approaches to identify non-coding RNAs. *Nucleic Acids Res* **34**, 635–646.

Hüttenhofer, A., Cavaille, J. & Bachellerie, J. P. (2004). Experimental RNomics: a global approach to identifying small nuclear RNAs and their targets in different model organisms. *Methods Mol Biol* **265**, 409–428.

Janzon, L. & Arvidson, S. (1990). The role of the δ-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. *EMBO J* **9**, 1391–1399.

Kawamoto, H., Koide, Y., Morita, T. & Aiba, H. (2006). Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol Microbiol* **61**, 1013–1022.

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

Komine, Y., Kitabatake, M., Yokogawa, T., Nishikawa, K. & Inokuchi, H. (1994). A tRNA-like structure is present in 10Sa RNA, a small stable RNA from *Escherichia coli*. *Proc Natl Acad Sci U S A* **91**, 9223–9227.

Lenz, D. H., Mok, K. C., Lilley, B. N., Kulkarni, R. V., Wingreen, N. S. & Bassler, B. L. (2004). The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae. Cell* **118**, 69–82.

Lin-Chao, S. & Bremer, H. (1986). Effect of the bacterial growth rate on replication control of plasmid pBR322 in *Escherichia coli. Mol Gen Genet* **203**, 143–149.

Livny, J., Brencic, A., Lory, S. & Waldor, M. K. (2006). Identification of 17 *Pseudomonas aeruginosa* sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2. *Nucleic Acids Res* **34**, 3484–3493.

Lung, B., Zemann, A., Madej, M. J., Schuelke, M., Techritz, S., Ruf, S., Bock, R. & Hüttenhofer, A. (2006). Identification of small non-coding RNAs from mitochondria and chloroplasts. *Nucleic Acids Res* **34**, 3842–3852.

Lynch, M. J., Drusano, G. L. & Mobley, H. L. (1987). Emergence of resistance to imipenem in *Pseudomonas aeruginosa. Antimicrob Agents Chemother* **31**, 1892–1896.

Massé, E. & Gottesman, S. (2002). A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli. Proc Natl Acad Sci U S A* **99**, 4620–4625.

Massé, E., Majdalani, N. & Gottesman, S. (2003). Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol* **6**, 120–124.

Mathews, D. H., Sabina, J., Zuker, M. & Turner, D. H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* **288**, 911–940.

McNealy, T. L., Forsbach-Birk, V., Shi, C. & Marre, R. (2005). The Hfq homolog in *Legionella pneumophila* demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA. *J Bacteriol* **187**, 1527–1532.

Miller, J. H. (1972). *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

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

Moll, I., Leitsch, D., Steinhauser, T. & Bläsi, U. (2003b). RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep* **4**, 284–289.

Møller, T., Franch, T., Udesen, C., Gerdes, K. & Valentin-Hansen, P. (2002). Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon. *Genes Dev* **16**, 1696–1706.

Nakao, H., Watanabe, H., Nakayama, S. & Takeda, T. (1995). *yst* gene expression in *Yersinia enterocolitica* is positively regulated by a chromosomal region that is highly homologous to *Escherichia coli* host factor 1 gene (*hfq*). *Mol Microbiol* **18**, 859–865.

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

Repoila, F., Majdalani, N. & Gottesman, S. (2003). Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli:* the RpoS paradigm. *Mol Microbiol* **48**, 855–861.

Rist, M. & Kertesz, M. A. (1998). Construction of improved plasmid vectors for promoter characterization in *Pseudomonas aeruginosa* and other Gram-negative bacteria. *FEMS Microbiol Lett* **169**, 179–183.

Robertson, G. T. & Roop, R. M., Jr (1999). The *Brucella abortus* host factor I (HF-I) protein contributes to stress resistance during stationary phase and is a major determinant of virulence in mice. *Mol Microbiol* **34**, 690–700.

Rose, D., Hertel, J., Reiche, K., Stadler, P. F. & Hackermüller, J. (2008). NcDNAlign: plausible multiple alignments of non-protein-coding genomic sequences. *Genomics* **92**, 65–74.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., No, R., Défago, G. & other authors (2000). Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J Bacteriol* **182**, 1215–1225.

Segal, R. & Ron, E. Z. (1996). Regulation and organization of the *groE* and *dnaK* operons in Eubacteria. *FEMS Microbiol Lett* **138**, 1–10.

Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H. & Mann, M. (1996). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc Natl Acad Sci U S A* **93**, 14440–14445.

Sittka, A., Pfeiffer, V., Tedin, K. & Vogel, J. (2007). The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium. Mol Microbiol* **63**, 193–217.

Sledjeski, D. D., Whitman, C. & Zhang, A. (2001). Hfq is necessary for regulation by the untranslated RNA DsrA. *J Bacteriol* **183**, 1997–2005.

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

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

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

Trias, J. & Nikaido, H. (1990). Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. *J Biol Chem* **265**, 15680–15684.

Ulanova, M., Petersen, T. D., Ciofu, O., Jensen, P., Hahn-Zoric, M., Hanson, L. A. & Hoiby, N. (1997). The clonal antibody response to *Pseudomonas aeruginosa* heat shock protein is highly diverse in cystic fibrosis patients. *APMIS* **105**, 449–456.

Valverde, C., Heeb, S., Keel, C. & Haas, D. (2003). RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Mol Microbiol* **50**, 1361–1379.

Van Delden, C. (2004). Virulence factors in *Pseudomonas aeruginosa.* In *Pseudomonas*, vol. 2, pp. 3–45. Edited by J. L. Ramos. New York: Kluwer Academic/Plenum Publishers.

Van Delden, C. & Iglewski, B. H. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* **4**, 551–560.

Vecerek, B., Moll, I., Afonyushkin, T., Kaberdin, V. & Bläsi, U. (2003). Interaction of the RNA chaperone Hfq with mRNAs: direct and indirect roles of Hfq in iron metabolism of *Escherichia coli. Mol Microbiol* **50**, 897–909.

Vogel, J., Bartels, V., Tang, T. H., Churakov, G., Slagter-Jager, J. G., Hüttenhofer, A. & Wagner, E. G. (2003). RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Res* **31**, 6435–6443.

Wadler, C. S. & Vanderpool, C. K. (2007). A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide. *Proc Natl Acad Sci U S A* **104**, 20454–20459.

Washietl, S. (2006). RNAz 1.0. Department for Theoretical Chemistry, University Vienna, 3, http://www.tbi.univie.ac.at/~wash/RNAz.

Washietl, S., Hofacker, I. L. & Stadler, P. F. (2005a). Fast and reliable prediction of noncoding RNAs. *Proc Natl Acad Sci U S A* **102**, 2454–2459.

Washietl, S., Hofacker, I. L., Lukasser, M., Hüttenhofer, A. & Stadler, P. F. (2005b). Mapping of conserved RNA secondary structures predicts thousands of functional noncoding RNAs in the human genome. *Nat Biotechnol* **23**, 1383–1390.

Wassarman, K. M., Repoila, F., Rosenow, C., Storz, G. & Gottesman, S. (2001). Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev* **15**, 1637–1651.

Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P. C., Gottesman, S., Ochsner, U. A. & Vasil, M. L. (2004). Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc Natl Acad Sci U S A* **101**, 9792–9797.

Williams, K. P. & Bartel, D. P. (1996). Phylogenetic analysis of tmRNA secondary structure. *RNA* **2**, 1306–1310.

Wilson, S. A. & Drew, R. E. (1995). Transcriptional analysis of the amidase operon from *Pseudomonas aeruginosa. J Bacteriol* **177**, 3052–3057.

Worhunsky, D. J., Godek, K., Litsch, S. & Schlax, P. J. (2003). Interactions of the non-coding RNA DsrA and RpoS mRNA with the 30 S ribosomal subunit. *J Biol Chem* **278**, 15815–15824.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

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

Zhang, A., Wassarman, K. M., Rosenow, C., Tjaden, B. C., Storz, G. & Gottesman, S. (2003). Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol* **50**, 1111–1124.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**, 3406–3415.

Edited by: L. S. Frost

4.2 The sRNA PaeII of *Pseudomonas aeruginosa* is induced upon nutrient limitation

Abstract

Small regulatory RNAs (sRNAs) are known to control various cellular processes including virulence gene expression in several bacterial pathogens. A bioinformatic approach employed to analyze intergenic regions of *Pseudomonas aeruginosa* for sRNAs revealed PaeII. PaeII expression is shown to be driven by a σ^{54}–dependent promoter, whereby vigorous synthesis occurs under nutrient limiting conditions. Evidence is presented that PaeII is processed by RNase E, whereby only the processed form is evolutionarily conserved in other *Pseudomonas* spp. The comparative analysis of the proteome profiles of the parental and the *P. aeruginosa paeII* deletion strain revealed an up-regulation of the open reading frames PA1579 and PA3332, whereas PA1244 and MvaT, encoding a H-NS family protein involved in virulence gene regulation, were down-regulated. In addition, a transcriptome analysis disclosed that the steady state levels of PA0740 (SdsA1) encoding a sodiumdodecylsulfate degrading sulfatase and PA4407, encoding a lactate permease decreased in the absence of *paeII*, whereas genes (PA3515-3519) involved in purine biosynthesis were found to increase at least two-fold.

Introduction

P. aeruginosa (PAO1) can adapt to a variety of environments, soil, water as well as to different hosts. As an opportunistic pathogen it can cause severe infections especially in immunocompromised patients. Due to its ability to form biofilms, PAO1 is able to escape host defence mechanisms and antibiotic treatment (Costerton *et al.*, 1999). Many virulence factors of PAO1 are controlled by three interlinked quorum sensing systems termed Las, Rhl and PQS, numerous two component systems as well as extra-cytoplasmatic sigma factors (for review van Delden and Iglewski, 1998).

Small regulatory RNAs (rRNAs) can modulate stress responses (i) by regulating the synthesis of the alternative sigma factor RpoS (Repoila *et al.*, 2003), (ii) through their function in iron metabolism (Massé *et al.*, 2007) and catabolite repression (Sonnleitner *et al.*, 2009) or (iii) by remodelling the outer membrane (Vogel and Papenfort, 2006). A large number of sRNAs associate with the RNA chaperone Hfq and require it to exert their function in post-transcriptional regulation (for review Valentin-Hansen *et al.*, 2004; Aiba, 2007; Brennan and Link, 2007; Waters and Storz, 2009). A PAO1*hfq*$^{-}$ mutant was attenuated in virulence, suggesting that sRNAs are involved in pathogenicity (Sonnleitner *et al.*, 2003). To date, the function of only a few PAO1 sRNAs has been studied in greater detail. Prrf1/2 are involved in maintaining iron homeostasis (Wilderman *et al.*, 2004; Oglesby *et al.*, 2008), whereas the GacA controlled RgsA appears to be involved in protection of PAO1 from oxidative stress (Gonzalez *et al.*, 2008). RsmY/Z and CrcZ sequester the translational repressor of virulence genes RsmA (Burrowes *et al.*, 2006; Kay *et al.*, 2006) and the translational repressor Crc involved in catabolite repression (Sonnleitner *et al.*, 2009), respectively. Moreover, approximately 30 sRNAs with unknown function have been recognized in PAO1, most of which have been predicted by biocomputation (Livny *et al.*, 2006; Gonzalez *et al.*, 2008; Sonnleitner *et al.*, 2008), while others were identified by RNomics (Sonnleitner *et al.*, 2008). Here, we analyzed the intergenic regions of PAO1 by

bioinformatics to expand the number of sRNAs in this pathogen. Among other sRNA candidates, the screen revealed the sRNA PaeII, which was further studied. First, we characterized the primary structure of PaeII and conditions that evoke *paeII* expression. Second, we identified regulators required for or involved in *paeII* transcription. Third, a proteome and a transcriptome analysis were performed to reveal potential target genes of PaeII.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. Unless indicated otherwise, bacterial cultures were grown at 37°C in Luria-Bertani (LB) medium (Miller, 1972), supplemented with appropriate antibiotics. BM2 minimal medium (40 mM K_2HPO_4, 22 mM KH_2PO_4, 7 mM $(NH4)_2SO_4$, 0.5 mM $MgSO_4$, 0.01 mM $FeSO_4$, 20 mM sugar (either succinate, mannitol, glutamate, glycerol or glucose, as indicated in the text) and M9 minimal medium supplemented with 20 mM glucose were used to mimic starvation, respectively. Antibiotics were added to a final concentration of 200 μg/ml spectinomycin-streptomycin, 50 μg/ml gentamicin, 125 μg/ml tetracyclin and 300 μg/ml carbenicillin for *P. aeruginosa* and 50 μg/ml gentamicin, 50 μg/ml tetracyclin and 100 μg/ml ampicillin for *E. coli*.

Bioinformatics

A search algorithm that follows the general scheme described by Lenz *et al.* (2004) was used to unravel novel sRNAs: (i) Most sRNAs are located in intergenic regions, therefore we restricted our search to regions between annotated genes. The *P. aeruginosa* intergenic regions were downloaded from http://www.pseudomonas.com (Intergenic DNA, *Pseudomonas aeruginosa* PAO1, version 2004-01-14). (ii) We assumed that the putative RNAs contain a Rho-independent terminator. 524 *P. aeruginosa* Rho-independent terminators were downloaded from http://www.tigr.org/software/transterm.html, of which 502 were found to be located in intergenic regions. (iii) The upstream region of the RNA loci were searched for a σ^{54}- (or a σ^{70}) binding site. Using PATSER (http://liv.bmc.uu.se:16080/rsa-tools, Pattern Matching, PATSER), the *P .aeruginosa* intergenic regions were screened for potential σ^{54}-binding sites with a weight matrix constructed from a compiled set of ~180 σ^{54}-binding sites from multiple bacterial species (Dombrecht *et al.,* 2002). All matches with a score higher than or equal to the cutoff score 8.9

(Dombrecht *et al.*, 2002) were considered as potential hits. The number of these 214 potential hits was further reduced to 168 by the assumption that the distance between the potential sRNAs and the next coding region should be at least 50 nucleotides. Combining these 168 hits with the list of Rho-independent terminators resulted in a list of 22 intergenic regions which are supposed to contain both a Rho-independent terminator and a σ^{54}-binding site. A final check of these 22 hits (the σ^{54}-binding site and the terminator sequences must have the same orientation - plus or minus, length and localization of the potential sRNAs within the intergenic region) left us with one sRNA candidate located in the intergenic region between the ORFs PA1781-PA1782.

A similar procedure was carried out to detect potential sRNAs carrying both a σ^{70}-binding site and a Rho-independent terminator. Again, the *P. aeruginosa* intergenic regions were screened with PATSER. We tested all of the best 11 weight matrices for the σ^{70}-binding site recognition (Huerta and Collado-Vides, 2003). The results were combined with the list of Rho-independent terminators and length and distance restrictions were applied. As a result of this procedure, we obtained four potential sRNA candidates, two of them are located in the intergenic region between the ORFs PA4704-PA4705, named PrrF1 and PrrF2 (Wilderman *et al.*, 2004), and the candidates in the intergenic region between the ORFs PA3535-PA3536 and the intergenic region between the ORFs PA4040-PA4041.

Creation of the *paeII* deletion mutant

To construct a PAO1 *paeII* deletion mutant, the sequence upstream (nucleotides -558 to -6 with regard to the transcriptional start) and the downstream sequence (nucleotides +79 to +680) of *paeII* were amplified by PCR using the oligonucleotide pairs Q_53 and R_53 and S_53 and T_53 (Table 2), respectively, and chromosomal PAO1 DNA as a template. The 602 nt long downstream fragment was cloned into the *PvuI* and *EcoRI* site of plasmid pSUP202, resulting in pSUP202-PaeIIdn. The 553 nt long upstream sequence was cloned into the *NdeI* and *PvuI* site of pSUP202-PaeIIdn, resulting in pSUP202-PaeIIko. This plasmid was transformed into the *E. coli*

strain S17-1 and transferred by conjugation to PAO1. The following homologous recombination resulted in the chromosomal deletion of *paeII*. The deletion was verified by PCR and Northern-blot analysis with gene-specific ^{32}P-end-labelled oligonucleotides (PaeII: V_28; 5S rRNA: I_26; Table 2).

Northern-Blot Analysis

Total RNA of PAO1 was purified using the hot phenol method (Lin-Chao and Bremer, 1986). The abundance of the RNAs was determined by Northern-blot hybridization using total RNA. The signals were normalized to the hybridization signal obtained for ribosomal 5S rRNA. The RNA samples were denatured for 5 min at 65°C in loading buffer, containing 50% formamide and then separated on 8% polyacrylamide (PAA) – 8 M urea gels followed by transfer to nylon membranes (Hybond N, GE-Healthcare) by electroblotting (Bio-Rad). The RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized with gene-specific ^{32}P-end-labelled oligonucleotides (PaeII: V_28; 5S rRNA: I_26; CrcZ: E_2; Table 2), and the hybridization signals were visualized using a PhosphoImager (Molecular Dynamics).

RpoN promoter-probe plasmid construction

To test for the *paeII* promoter activity the plasmids pMEPaeII, pMEPaeII-RpoN1 and pMEPaeII-RpoN5 were constructed. The respective DNA sequence of *paeII*, including its authentic promoter was amplified by primer pairs K_29 and L_29 (Table 2) and blunt-end ligated into the *SmaI* site of vector pME4510. The forward primer, containing the mutated *paeII* promoter region, Y_57 (Table 2) was used for PCR together with reverse primer L_29 (Table 2), followed by ligation of the PCR fragments into *SmaI* restriction site of pME4510 resulting in plasmid pMEPaeII-RpoN5, respectively. The PaeII levels were analyzed by Northern-blot analysis with gene-specific ^{32}P-end-labelled oligonucleotides (PaeII: V_28; 5S rRNA: I_26; Table 2). The PaeII transcript levels present in strain PAO1Δ*paeII* (pME-PaeII) was set as 100%.

Expression of *paeII* under different stress conditions

PAO1 cultures were grown in LB medium to an OD_{600} of 1.0 in LB medium at 37°C and then exposed to 43°C for 15 min (heat shock), to 15°C for 30 min (cold shock), to 1mM hydrogen-peroxide for 15 min (oxidative stress), to 500 µM dipyridyl for 15 min (iron deprivation), to 0.3 M NaCl for 15 min (osmotic shock) or to HCl, pH=3 for 15 min (low pH). To mimic starvation, PAO1 was grown in BM2 minimal medium (40 mM K_2HPO_4, 22 mM KH_2PO_4, 7 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 0.01 mM $FeSO_4$) supplemented with 20 mM succinate to an OD_{600} of 1.0. After those treatments, total RNA was isolated and 10 µg of the total RNA were subjected to Northern-blot analysis (PaeII: V_28; 5S rRNA: I_26; Table 2).

RNA preparation

PaeII and RsmY RNA were *in vitro* transcribed with T7 RNA polymerase (Epicentre AmpliScribe™ T7-*Flash*™ Transcription Kit) from PCR templates. The *paeII* PCR fragment was amplified using the forward primer A_57 and the reverse primer L_29 (Table 2). For RsmY transcription the oligonucleotides U_26 and V_26 (Table 2) were used as previously described (Sonnleitner *et al.*, 2006). The forward primers A_57 and U_26 contained a T7Φ10 promoter sequence. After *in vitro* transcription the RNAs were purified on a 6% polyacrylamide-8 M urea gel, and the RNA concentration was determined by measuring the absorbance at 260 nm.

Hfq binding assay

Gel purified PaeII RNA was 5'-end labelled with [γ-^{32}P]-ATP (Hartmann), and re-purified on a 6% polyacrylamide-8M urea gel as described (Sonnleitner *et al.*, 2006). Labelled RNA (0.05 pmol) was first incubated at room temperature for 5 min, and then for 15 min with or without increasing concentrations of purified Hfq-hexamer (Hfq_6) (0.1, 0.2, 0.4, 0.8, 0.16 or 0.32 pmol) in 10 µl VD buffer (10 mM Tris, pH 7.4, 60 mM NH_4Cl, 6 mM β-mercaptoethanol and 2 mM

Mg-acetate). 0.25 and 1 pmol PaeII, RsmY and tRNA were added as competitors to labelled PaeII RNA. The samples were mixed with 4 µl of loading dye (75% glycerol, 0,01% xylencyanol, 0,01% bromphenol blue) and then separated on a native 4% polyacrylamide gel. Electrophoresis was performed in TAE buffer at 160 V at 4°C. The radioactively labelled bands were visualized with a PhosphoImager (Molecular Dynamics).

Mapping of the 5´and 3´termini of PaeII

Simultanous mapping of the 5' and 3' ends of PaeII was performed as previously described (Toledo-Arana *et al.*, 2009). 6 µg of total RNA was incubated for 45 min at 37ºC with the corresponding buffer, in the presence or absence of Tobacco Acid Pyrophosphatase (TAP) (Epicentre biotechnologies). This step allows to distinguish between a 5'-end generated by transcription initiation and a 5'-end resulting from endonucleolytic processing. It was therefore omitted to map the *paeII* processing site. After phenol and chloroform extraction and ethanol precipitation, serial dilutions (from 500 ng to 0.5 ng) of the TAP+ and TAP- treated RNAs were prepared. Each sample was ligated with 40 U of T4 RNA ligase I (Fermentas) in the presence of 1x RNA ligase buffer supplemented with 8% DMSO, 10 U of RNase Inhibitor, 1 U of DNase I and RNase-free water in a total volume of 25 µl at 37ºC for 1 h. After phenol and chloroform extraction and ethanol precipitation, the ligated RNAs were resuspended in 10 µl of RNase-free water. The RT-PCR reactions were performed using specific outward primers V_51 and W_51 (Table 2) and the SuperScript One-Step RTPCR kit (Invitrogen). Bands only present in the TAP+ reactions with the expected size were purified using the Gel Extraction kit (QIAGEN) and cloned using the pGEM®-T Easy Vector System (Promega). 10 plasmid clones containing the expected size of insert were sequenced. The sequences were compared with the *P. aeruginosa* genome (www.pseudomonas.com) to localize the 5'- and 3'-ends.

Determination of PaeII stability and abundance

The steady state levels and the stability of PaeII were determined in strains PAO1 and PAO1*hfq*¯. At an OD_{600} of 1, rifampicin (500 µg/µl) was added and 4 ml aliquots were withdrawn 0, 2.5, 5, 10 and 20 min thereafter for isolation of total RNA. Total RNA was purified by the hot phenol method (Lin-Chao and Bremer, 1986). The PaeII concentrations were determined by Northern-blot hybridization, using the 5'-end labeled oligonucleotide V_28 for PaeII (Table 2). The 5S rRNA was used as an internal control and was detected with primer I_26 (Table 2).

RNase E cleavage

0.15 pmol of 5'-[^{32}P] labelled PaeII RNA was incubated with hexameric Hfq (Hfq_6) (0.3 pmol or 0,6 pmol) for 15 min at room temperature. PaeII cleavage was carried out using 25 ng of the purified N-terminal fragment of *E. coli* RNase E (Rne498) (Kaberdin *et al.*, 2000) in VD buffer in the presence of 5 mM EGTA. The cleavage reaction was started by addition of MgOAc to a final concentration of 5 mM, and was continued for 30 min at 37°C. The cleavage reaction was terminated by addition of 1µl of 1µg/µl glycogen in 250 mM EDTA solution. Sequence ladders were obtained by RNase A and T1 digestion under denaturing conditions as described (Kuchino and Nishimura, 1989). After phenol extraction, the RNA was precipitated and analyzed on a 10% polyacrylamide-8M urea gel. The radioactively labelled bands were visualized using a PhosphoImager (Molecular Dynamics).

Proteomics

To detect PaeII-mediated regulatory effects, the protein patterns of total cellular extracts of strain PAO1 and PAO1Δ*paeII* were compared by two-dimensional gel electrophoresis. The strains were grown in BM2 minimal medium containing mannitol to an OD_{600} of 1, and equal amounts of cells were dissolved in lysis buffer (8 M urea, 4% (w/v) CHAPS and 40 mM Tris-base). The cells were disrupted by repeated freezing in liquid N_2 and thawing at 37°C followed by

incubation at 37°C for 1 h to achieve complete lysis. For the first dimension, the Immobiline Dry Strip pH 3–10 (18 cm; Amersham Pharmacia Biotech) was used with the following IEF program: 12 h rehydration at 40 V, 0.5 h at 300 V, 0.5 h at 1000 V, 0.5 h at 2000 V, 0.5 h at 3000 V, 9 h at 7000 V at 18°C (IPGphor isoelectric focusing system). The resolution in the second dimension was performed on 12% SDS-polyacryamide gels for 15 min at 15 mA, and then for 4 h at 20 mA. Buffers and conditions were used according to the manufacturer's instructions. The gels were silver-stained as described previously (Shevchenko *et al.*, 1996). Selected protein spots with different intensities were excised from the gel to assess the protein identities by mass spectrometry.

DNA microarray studies

RNA was isolated from 10 ml of PAO1 and PAO1*ΔpaeII* cultures grown in BM2 minimal medium supplemented with mannitol to an OD_{600} of 1. A total of 1 x 10^9 cells were mixed with RNA Protect Bacteria Reagent (Quiagen) and processed as recommended by the manufacturer. 30 minutes after induction total RNA treated with RNA protect bacteria reagent® (Qiagen) was purified by using the hot acid phenol method as described above. The integrity of total RNA was confirmed by agarose gel electrophoresis and an RNA 6000 Nano LabChip in an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA isolation was tested for residual DNA by PCR with oligonucleotides specific for 16S rRNA (L_37 and M_37, Table 2) and *ladS* (O_45 and N_45, Table 2). For the microarray analysis *P. aeruginosa* GeneChip arrays (Affymetrix, Inc., Santa Clara, CA) were used. cDNA synthesis, fragmentation, labeling, hybridization, staining, and washing steps were performed according to the manufacturer's protocol for the Affymetrix *P. aeruginosa* GeneChip arrays at the University of Lausanne, Center for Integrative Genomics, as described previously (Frangipani *et al.*, 2008). For each condition, cultures were grown in triplicate, and RNAs from these cultures were pooled before proceeding to cDNA synthesis. In addition, biological replicates for each condition were performed on a

separate day and run on a different microarray chip. The expression of *paeII* under these condition was verified by Northern blotting as described above with gene-specific ^{32}P-end-labelled oligonucleotides (PaeII: V_28; 5S rRNA: I_26; Table 2).

Results and Discussion

Mapping of the 5´and 3´ termini of PaeII.

The bioinformatic search based on the prediction of (i) a putative promoter, (ii) a Rho-independent terminator, and (iii) a length between 50 and 300 nucleotides (nts) as well as (iv) the location in an intergenic region revealed the sRNA candidate PaeII, which is encoded between the non-characterized open reading frames PA4040 and PA4041 (Stover *et al.*, 2000). PaeII is predicted to contain three stem-loop structures. The 3´- terminal stem-loop structure, which is followed by six consecutive U's, most likely serves as a transcriptional terminator (Fig. 1A). PaeII was estimated to be 108 nt in length, which was in agreement with the size of the corresponding larger signal detected by Northern-blot analysis (Fig. 1B). In addition to the full length PaeII signal, a stronger signal with an approximate size of ~70 nt was as well detected (Fig. 1B). To map the 5´and 3´ termini of PaeII, total RNA isolated from PAO1 was circularized, amplified, cloned and sequenced. This analysis revealed that the full length PaeII sRNA consists of 108 nts (Fig. 1C). Circularization of RNA entails removal of the 5'-phosphate by Tobacco acid pyrophosphatase (TAP) treatment. However, when the RNA was processed by an endonuclease, the 5´-phosphate is removed and TAP treatment is dispensable for circularization. When TAP treatment was omitted before circularization, the 3' end was mapped to nt position +33 (Fig. 1C, open arrow). Thus, nuclease cleavage at nt position 33 could explain the appearance of the 76 nt long processed fragment (Fig. 1B). The putative processing site in PaeII is AU-rich and located in a single stranded region adjacent to stem-loop structures (Fig. 1A and 1C). As this motif complies with the consensus recognition motif for RNAse E in *E. coli* (Kaberdin, 2003), and *E. coli* RNase E has been used to mimic *P. aeruginosa* mRNA processing (Gamper and Haas, 1993), we next used the catalytic fragment of purified RNase E to cleave PaeII RNA *in vitro*. RNase E cleavage assays were performed with ^{32}P-end-labelled *paeII* RNA. Two predominant RNAse E cleavage sites were detected in the AU-rich single stranded region

between the two stem-loop structures (Fig. 2). In fact, cleavage site 1 coincided with the 5' end which was mapped by 3'-5' circularization (Fig. 1C, open arrow).

A BlastN search (Altschul *et al.*, 1997) with the *paeII* sequence against the sequenced genomes of different pseudomonads revealed that only the 3´-terminal part of *paeII* is conserved in *P. mendocina, P. entomophila, P. stutzeri, P. putida* and in *P. fluorescens*. The 5´ terminal part of *paeII*, i.e. the first 32 nt are only conserved in the *P. aeruginosa* strains PAO1, PA14, LESB58 and PA7 (Fig. 1D). Therefore, the bioinformatic prediction of PaeII was possible because -in contrast to other bioinformatical approaches (Livny *et al.*, 2006; Gonzalez *et al.*, 2008)- sequence conservation was not a primary search criterium. In some pseudomonads the *paeII* homologous sequences overlap with ORFs. In *P. fluorescens* Pf-5 the *paeII* homologous sequence (genome position: 6251515- 6251587) (Paulsen *et al.*, 2005) overlaps for the first 72 nt with an open reading frame encoding the putative lipoprotein PFL5479 (genome position: 6251196-6251585) (Paulsen *et al.*, 2005). In *P. stutzeri* it overlaps for a stretch of 26 nt with the sequence encoding an inorganic pyrophosphatase (*ppa-1*, genome position: 3985344- 3985370) (Yan *et al.*, 2008) and in *P. mendocina ymp* for a stretch of 40 nt with an inorganic pyrophosphatase (genome position: 4217394-4217420, http://genome.jgi-psf.org/pseme/pseme.home.html).

Nutrient limitation induces *paeII* expression.

Non-coding RNAs are known to regulate different metabolic processes and many of them are expressed as a response to certain stress conditions. To assess conditions that trigger *paeII* expression, different stress conditions were applied including heat-, cold-, osmotic-, pH-, iron-, stationary phase- and oxidative- stress as well as nutrient limitation. A massive induction of *paeII* expression was observed when PAO1 was grown in BM2 minimal medium supplemented with succinate and the majority of PaeII was found to be processed to the shorter transcript (Fig. 3A, lane 2). All other tested stress conditions had no major impact on *paeII* transcription (Fig. 3A). PaeII is transcribed throughout growth, but peaked upon entry into stationary phase (Fig. 3B).

Further analyses revealed only slight effects of the carbon source on *paeII* expression. When compared to rich medium (LB), other single carbon sources, like mannitol, glycerol or glucose similarly stimulated *paeII* expression (Fig. 3C). Even though *paeII* expression is massively induced in minimal medium it is not essential for bacterial survival or growth, since growth of the *paeII* mutatant was not impaired in minimal medium (data not shown).

Two promoters drive *paeII* transcription

As shown in Fig. 1C the *paeII* gene is preceded by a putative σ^{70} as well as by a putative σ^{54} dependent promoter. To test whether RpoN is required for *paeII* transcription we first compared the steady state levels of PaeII in PAO1 with that in a PAO1Δ*rpoN* mutant strain. As shown in Fig. 4A, when compared to PAO1 the steady state levels of *paeII* were reduced by ~70% in the PAO1Δ*rpoN* strain. To verify the putative σ^{54} dependent promoter several mutations (Fig. 1C) were introduced in the -12 and -24 region (Fig. 1C) and the PaeII levels obtained with the plasmid borne *paeII* gene were assessed in the PAO1Δ*paeII* strain by Northern-blot analysis. As shown in Fig. 4B, the mutations in the putative σ^{54} dependent promoter resulted in a significant decrease of the *paeII* levels. The PaeII steady state levels decreased to 20% (Fig. 4B), which is similar to the amount of PaeII remaining in an *rpoN* mutant strain (Fig. 4A), and may be explained by transcription from the σ^{70} dependent promoter.

Bacteria respond to starvation through the stringent response, i.e. by synthesis of the alarmone ppGpp. In *E. coli* the level of ppGpp is controlled by two enzymes RelA, which responds to amino-acid starvation (Xiao *et al.*, 1991), and SpoT, which responds to nutrient and micronutrient starvation, e.g. carbon-, phosphate- or iron starvation (Magnusson *et al.*, 2005). Orthologues of both, *spoT* and *relA*, are present in PAO1 (van Delden *et al.*, 2001). High level transcription from the σ^{54}–dependent Po promoter in both *E. coli* (Bernardo *et al.*, 2006) and *P. putida* (Bernardo *et al.*, 2009) requires the bacterial alarmone ppGpp and DksA. Preliminary data

based on a transcriptome analysis shows that the *spoT* steady state levels were two-fold decreased upon *rpoN* deletion when cells were grown in LB medium (E. Sonnleitner, unpublished observation). Thus, it appears that RpoN controls *spoT* expression, but it remains to be shown if this is also the case under conditions when RpoN and/or SpoT are required. Most likely, the observed up-regulation from a σ^{54}–dependent promoter during the stringent response is indirect and results from an increase in the quantity of the core RNA polymerase (RNAP), which is available to form the σ^{54}–RNAP holoenzyme. The vigorous induction of *paeII* transcription observed during slow growth in minimal medium (Fig. 3A), i.e. under conditions when ppGpp levels are anticipated to increase, would be in line with σ^{54}–dependent expression of *paeII*. To support our hypothesis that *paeII* transcription is predominantly achieved by σ^{54}–RNAP we examined the PaeII levels in PAO1 *relA,* and *relA*/*spoT* mutant strains. When compared to PAO1, the PaeII levels were slightly decreased in the Δ*relA* strain NB159, whereas a strong decrease was observed in the *ΔrelA/ΔspoT* double mutant NB170 (Fig. 4C). Similarly, a significant decrease of the steady state levels of the sRNA CrcZ (used as a variation control), the expression of which is known to be σ^{54}-dependent (Sonnleitner *et al.*, 2009), was observed in strain NB170 (Fig. 4C).

Taken together, the *paeII* expression studies revealed that this sRNA is primarily synthesized under nutrient limitation in a manner dependent on σ^{54}. In *E. coli* one example is known where a sRNA is transcribed from both, a σ^{70} or σ^{54} dependent promoter (Reichenbach *et al.*, 2009). In this case both promoters overlap in a way that the corresponding transcriptional start sites map to the same position (Reichenbach *et al.*, 2009), which seems to be also the case for *paeII* (Fig. 1C). This regulation would ensure a basal expression level during exponential growth dependent on σ^{70}, which can increase upon activation of the σ^{54}- dependent promoter.

PaeII steady state levels are decreased in a PAO1*hfq*$^-$ strain

As Hfq has been shown to affect the levels of some sRNAs in PAO1 (Sonnleitner *et al.*, 2006; Sonnleitner *et al.*, 2008), we next tested whether the RNA chaperone binds to and affects PaeII stability and/or abundance. The band-shift assays shown in Supplementary Fig. S1 suggested that Hfq binds to PaeII, albeit with a rather low affinity. PaeII and the competitor RsmY (Sonnleitner *et al.*, 2006) were able to compete efficiently for Hfq binding (Supplementary Fig. S1, lane 8 and 10), whereas the non-specific competitor tRNA was not. Using band-shift assays it proved to be difficult to determine the affinity of Hfq for PaeII. With the exception of the B complex (Supplementary Fig. S1) a smear was constantly seen when increasing amounts of Hfq were added to the RNA and higher molecular weight complexes appeared to be retained in the wells. Whether the observed phenomenon is due to Hfq and/or RNA aggregation was not further studied.

Next, we tested whether the stability of PaeII depends on Hfq as shown for the PAO1 RsmY RNA (Sonnleitner *et al.*, 2006). Both strains, PAO1 and PAO1*hfq*$^-$ were grown in BM2 minimal medium supplemented with 20 mM mannitol to an OD_{600} of 1, when rifampicin was added. Samples for RNA isolation were withdrawn before and several times after addition of the antibiotic. As shown in Fig. 5A and 5C, Hfq had no major impact on the stability of PaeII. Similarly, as shown before for the PAO1 sRNA PhrS (Sonnleitner *et al.*, 2008), the abundance of PaeII was decreased in the absence of Hfq (Fig. 5B). As Hfq and RNase E binding sites can coincide (Moll *et al.*, 2003; Sorger-Domenigg *et al.*, 2007) we also tested whether Hfq can protect PaeII from processing by RNase E *in vitro*. As shown in Fig. 2 (lane 7 and 8) RNase E-dependent processing of *paeII* was not affected by the presence of Hfq. Taken together these results suggest that Hfq affects *paeII* transcription. Since a transcriptome analysis did not reveal any regulatory effect of Hfq on the *rpoN* gene and the *spoT* levels were unaltered in a *hfq* mutant (Sonnleitner *et al.*, 2006), these data further suggest that Hfq-mediated expression of *paeII* is indirect.

Potential targets of PaeII revealed by proteomics and transcriptomics

Given the strong induction of *paeII* expression under nutrient limitation we compared the proteome profiles of strain PAO1 and PAO1Δ*paeII* grown in BM2 minimal medium supplemented with 20 mM mannitol to reveal potential targets of PaeII. Four spots showing different intensities in the 2D-gels were analyzed by mass-spectrometry to reveal their identity. The two proteins showing higher abundance in the presence of PaeII were identified as encoded by ORF PA1244 (Fig. 6, circle 1) and by the *mvaT* gene (Fig. 6, circle 3). PA1244 codes for a protein with unknown function and and *mvaT* for a transcriptional regulator (Diggle *et al.*, 2002). MvaT belongs to the HN-S family of transcriptional regulators that binds to AT-rich regions, which results in repression through their DNA-binding- and possible DNA-bridging activities and they coordinately function as xenogeneic silencers in *P. aeruginosa* (Castang *et al.*, 2008). MvaT controls multiple functions including those involved in arginine metabolism, pyocyanin synthesis and prophage activation (Li *et al.*, 2009). However, no changes in pyocyanin production were obvious between PAO1 and PAO1Δ*paeII* (data not shown). Among the proteins that were induced in the absence of PaeII was PA1579 (Fig. 6, circle 2), a protein with unknown function, which is exported across the inner membrane (Lewenza *et al.*, 2005) and PA3332 (Fig. 6, circle 4), an uncharacterized protein belonging to the family of phenanzine biosynthesis genes *phzA/B*, whose 3D-structure has been solved (RCSB PDB ID: 1Z1S).

With the reasoning that a stimulating effect of a sRNA on translation generally results in stabilization and that inhibition of translation by a sRNA results generally in destabilization of the target mRNA we compared the transcriptome profiles of PAO1 and PAO1Δ*paeII* grown in BM2 minimal medium supplemented with 20 mM mannitol. The overall number of genes that were significantly affected was surprisingly low, with two genes being up-regulated and seven being down-regulated in the presence of PaeII (Table 3). For the proteins that were detected by proteomics no alterations in the transcriptome levels could be detected. Thus, more studies are

needed to dicern whether PaeII regulates the identified proteins in a direct or indirect manner. The most down-regulated genes PA3515-3519 appear to form an operon (www.pseudomonas.com). While the function of PA3515, PA3518 and PA3519 is unknown, PA3516 and PA3517 are predicted to encode hypothetical lyases, which are linked to purine biosynthesis. Both are predicted to have an adenylosuccinate lyase activity which converts andenylosuccinate to AMP and fumarate during purine ribonucleotide biosynthesis. We speculate that a down-regulation of *de novo* purine biosynthesis under starvation conditions allows *P. aeruginosa* to maintain the expression of essential genes at the expense of DNA replication. The alarmone ppGpp is also known to control transcription and replication at different levels (Srivatsan and Wang, 2008) and thereby could indirectly downregulate purine synthesis *via* PaeII. No reduction of the growth rate was observed in the absence of the sRNA in BM2 minimal medium (data not shown). PA3520 (Table 3), which was ~2-fold down-regulated in the presence of PaeII was likewise found to be down-regulated in a *rhlR lasR* double mutant. Thus, it seems to be activated by quorum sensing, which is consistent with a putative *las-rhl* box-like sequence upstream of the gene (Schuster *et al.*, 2003). The most up-regulated gene in the presence of PaeII was PA0740, which has been identified as a zinc-dependent, secreted sulfatase. This enzyme allows PAO1 to utilize primary sulfates such as sodiumdodecysulfate (SDS) as a sole carbon and energy source (Hagelueken *et al.*, 2006). A PA0740 mutant was shown to be attenuated in killing of *Caenorhabditis elegans* (Wiehlmann *et al.*, 2007) and in the degradation of acyl-homoserine lactones (Adams, 2004). The other up-regulated gene PA4407 (Table 3), a lactate permease, was down-regulated in a *rhlR lasR* mutant, indicating that it is quorum-repressed (Schuster *et al.*, 2003). The effect of PaeII on these two proteins may be explained in a way that PaeII somehow senses nutrient starvation and subsequently induces the expression of genes that are needed for the uptake and usage of these alternative energy sources.

References

Adams, T. (2004). "Funktionelle Charakterisierung potentieller Pathogenitätsfaktoren aus *Pseudomonas aeruginosa* mittels biochemischer und evolutiver Methoden." Göttingen, Univ., Diss.

Aiba, H. (2007). "Mechanism of RNA silencing by Hfq-binding small RNAs." Curr Opin Microbiol **10**(2): 134-9.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res **25**(17): 3389-402.

Bernardo, L. M., L. U. Johansson, D. Solera, E. Skarfstad and V. Shingler (2006). "The guanosine tetraphosphate (ppGpp) alarmone, DksA and promoter affinity for RNA polymerase in regulation of sigma-dependent transcription." Mol Microbiol **60**(3): 749-64.

Bernardo, L. M., L. U. Johansson, E. Skarfstad and V. Shingler (2009). "sigma54-promoter discrimination and regulation by ppGpp and DksA." J Biol Chem **284**(2): 828-38.

Boes, N., K. Schreiber and M. Schobert (2008). "SpoT-triggered stringent response controls *usp* gene expression in *Pseudomonas aeruginosa*." J Bacteriol **190**(21): 7189-99.

Brennan, R. G. and T. M. Link (2007). "Hfq structure, function and ligand binding." Curr Opin Microbiol **10**(2): 125-33.

Burrowes, E., C. Baysse, C. Adams and F. O'Gara (2006). "Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis." Microbiology **152**(Pt 2): 405-18.

Castang, S., H. R. McManus, K. H. Turner and S. L. Dove (2008). "H-NS family members function coordinately in an opportunistic pathogen." Proc Natl Acad Sci U S A **105**(48): 18947-52.

Costerton, J. W., P. S. Stewart and E. P. Greenberg (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-22.

Diggle, S. P., K. Winzer, A. Lazdunski, P. Williams and M. Camara (2002). "Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of N-acylhomoserine lactone production and virulence gene expression." J Bacteriol **184**(10): 2576-86.

Dombrecht, B., K. Marchal, J. Vanderleyden and J. Michiels (2002). "Prediction and overview of the RpoN-regulon in closely related species of the Rhizobiales." Genome Biol **3**(12): RESEARCH0076.

Frangipani, E., V. I. Slaveykova, C. Reimmann and D. Haas (2008). "Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation." J Bacteriol **190**(20): 6706-17.

Gamper, M. and D. Haas (1993). "Processing of the *Pseudomonas arcDABC* mRNA requires functional RNase E in *Escherichia coli*." Gene **129**(1): 119-22.

Gonzalez, N., S. Heeb, C. Valverde, E. Kay, C. Reimmann, T. Junier and D. Haas (2008). "Genome-wide search reveals a novel GacA-regulated small RNA in *Pseudomonas* species." BMC Genomics **9**: 167.

Hagelueken, G., T. M. Adams, L. Wiehlmann, U. Widow, H. Kolmar, B. Tümmler, D. W. Heinz and W. D. Schubert (2006). "The crystal structure of SdsA1, an alkylsulfatase from *Pseudomonas aeruginosa*, defines a third class of sulfatases." Proc Natl Acad Sci U S A **103**(20): 7631-6.

Heurlier, K., V. Denervaud, G. Pessi, C. Reimmann and D. Haas (2003). "Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1." J Bacteriol **185**(7): 2227-35.

Holloway, B. W., V. Krishnapillai and A. F. Morgan (1979). "Chromosomal genetics of *Pseudomonas*." Microbiol Rev **43**(1): 73-102.

Huerta, A. M. and J. Collado-Vides (2003). "Sigma70 promoters in *Escherichia coli*: specific transcription in dense regions of overlapping promoter-like signals." J Mol Biol **333**(2): 261-78.

Kaberdin, V. R., A. P. Walsh, T. Jakobsen, K. J. McDowall and A. von Gabain (2000). "Enhanced cleavage of RNA mediated by an interaction between substrates and the arginine-rich domain of *E. coli* ribonuclease E." J Mol Biol **301**(2): 257-64.

Kaberdin, V. R. (2003). "Probing the substrate specificity of *Escherichia coli* RNase E using a novel oligonucleotide-based assay." Nucleic Acids Res **31**(16): 4710-6.

Kay, E., B. Humair, V. Denervaud, K. Riedel, S. Spahr, L. Eberl, C. Valverde and D. Haas (2006). "Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*." J Bacteriol **188**(16): 6026-33.

Kuchino, Y. and S. Nishimura (1989). "Enzymatic RNA sequencing." Methods Enzymol **180**: 154-63.

Lenz, D. H., K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen and B. L. Bassler (2004). "The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*." Cell **118**(1): 69-82.

Lewenza, S., J. L. Gardy, F. S. Brinkman and R. E. Hancock (2005). "Genome-wide identification of *Pseudomonas aeruginosa* exported proteins using a consensus computational strategy combined with a laboratory-based PhoA fusion screen." Genome Res **15**(2): 321-9.

Li, C., H. Wally, S. J. Miller and C. D. Lu (2009). "The multifaceted proteins MvaT and MvaU, members of the H-NS family, control arginine metabolism, pyocyanin synthesis, and prophage activation in *Pseudomonas aeruginosa* PAO1." J Bacteriol **191**(20): 6211-8.

Lin-Chao, S. and H. Bremer (1986). "Effect of the bacterial growth rate on replication control of plasmid pBR322 in *Escherichia coli*." Mol Gen Genet **203**(1): 143-9.

Livny, J., A. Brencic, S. Lory and M. K. Waldor (2006). "Identification of 17 *Pseudomonas aeruginosa* sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2." Nucleic Acids Res **34**(12): 3484-93.

Magnusson, L. U., A. Farewell and T. Nystrom (2005). "ppGpp: a global regulator in *Escherichia coli*." Trends Microbiol **13**(5): 236-42.

Massé, E., H. Salvail, G. Desnoyers and M. Arguin (2007). "Small RNAs controlling iron metabolism." Curr Opin Microbiol **10**(2): 140-5.

Miller, J. H. (1972). "Experiments in Molecular Genetics." Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Moll, I., T. Afonyushkin, O. Vytvytska, V. R. Kaberdin and U. Bläsi (2003). "Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs." RNA **9**(11): 1308-14.

Nikulin, A., E. Stolboushkina, A. Perederina, I. Vassilieva, U. Bläsi, I. Moll, G. Kachalova, S. Yokoyama, D. Vassylyev, M. Garber and S. Nikonov (2005). "Structure of *Pseudomonas aeruginosa* Hfq protein." Acta Crystallogr D Biol Crystallogr **61**(Pt 2): 141-6.

Oglesby, A. G., J. M. Farrow, 3rd, J. H. Lee, A. P. Tomaras, E. P. Greenberg, E. C. Pesci and M. L. Vasil (2008). "The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing." J Biol Chem **283**(23): 15558-67.

Paulsen, I. T., C. M. Press, J. Ravel, D. Y. Kobayashi, G. S. Myers, D. V. Mavrodi, R. T. DeBoy, R. Seshadri, Q. Ren, R. Madupu, R. J. Dodson, A. S. Durkin, L. M. Brinkac, S. C. Daugherty, S. A. Sullivan, M. J. Rosovitz, M. L. Gwinn, L. Zhou, D. J. Schneider, S. W. Cartinhour, W. C. Nelson, J. Weidman, K. Watkins, K. Tran, H. Khouri, E. A. Pierson, L. S. Pierson, 3rd, L. S. Thomashow and J. E. Loper (2005). "Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5." Nat Biotechnol **23**(7): 873-8.

Reichenbach, B., Y. Gopel and B. Görke (2009). "Dual control by perfectly overlapping sigma 54- and sigma 70-promoters adjusts small RNA GlmY expression to different environmental signals." Mol Microbiol **74**(5): 1054-70.

Repoila, F., N. Majdalani and S. Gottesman (2003). "Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm." Mol Microbiol **48**(4): 855-61.

Rist, M. and M. A. Kertesz (1998). "Construction of improved plasmid vectors for promoter characterization in *Pseudomonas aeruginosa* and other gram-negative bacteria." FEMS Microbiol Lett **169**(1): 179-83.

Schuster, M., C. P. Lostroh, T. Ogi and E. P. Greenberg (2003). "Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis." J Bacteriol **185**(7): 2066-79.

Shevchenko, A., O. N. Jensen, A. V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, H. Boucherie and M. Mann (1996). "Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels." Proc Natl Acad Sci U S A **93**(25): 14440-5.

Simon, R., U. Priefer and A. Puhler (1983). "A Broad Host Range Mobilization System for Invivo Genetic-Engineering - Transposon Mutagenesis in Gram-Negative Bacteria." Bio-Technology **1**(9): 784-791.

Simon, R., M. O'Connell, M. Labes and A. Puhler (1986). "Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria." Methods Enzymol **118**: 640-59.

Sonnleitner, E., S. Hagens, F. Rosenau, S. Wilhelm, A. Habel, K. E. Jäger and U. Bläsi (2003). "Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1." Microb Pathog **35**(5): 217-28.

Sonnleitner, E., M. Schuster, T. Sorger-Domenigg, E. P. Greenberg and U. Bläsi (2006). "Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*." Mol Microbiol **59**(5): 1542-58.

Sonnleitner, E., T. Sorger-Domenigg, M. J. Madej, S. Findeiss, J. Hackermüller, A. Hüttenhofer, P. F. Stadler, U. Bläsi and I. Moll (2008). "Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools." Microbiology **154**(Pt 10): 3175-87.

Sonnleitner, E., L. Abdou and D. Haas (2009). "Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*." Proc Natl Acad Sci U S A.

Sorger-Domenigg, T., E. Sonnleitner, V. R. Kaberdin and U. Bläsi (2007). "Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY." Biochem Biophys Res Commun **352**(3): 769-73.

Srivatsan, A. and J. D. Wang (2008). "Control of bacterial transcription, translation and replication by (p)ppGpp." Curr Opin Microbiol **11**(2): 100-5.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory and M. V. Olson (2000). "Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen." Nature **406**(6799): 959-64.

Suh, S. J., L. Silo-Suh, D. E. Woods, D. J. Hassett, S. E. West and D. E. Ohman (1999). "Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*." J Bacteriol **181**(13): 3890-7.

Toledo-Arana, A., O. Dussurget, G. Nikitas, N. Sesto, H. Guet-Revillet, D. Balestrino, E. Loh, J. Gripenland, T. Tiensuu, K. Vaitkevicius, M. Barthelemy, M. Vergassola, M. A. Nahori, G. Soubigou, B. Regnault, J. Y. Coppee, M. Lecuit, J. Johansson and P. Cossart (2009). "The *Listeria* transcriptional landscape from saprophytism to virulence." Nature **459**(7249): 950-6.

Totten, P. A., J. C. Lara and S. Lory (1990). "The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene." J Bacteriol **172**(1): 389-96.

Valentin-Hansen, P., M. Eriksen and C. Udesen (2004). "The bacterial Sm-like protein Hfq: a key player in RNA transactions." Mol Microbiol **51**(6): 1525-33.

van Delden, C. and B. H. Iglewski (1998). "Cell-to-cell signaling and *Pseudomonas aeruginosa* infections." Emerg Infect Dis **4**(4): 551-60.

van Delden, C., R. Comte and A. M. Bally (2001). "Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*." J Bacteriol **183**(18): 5376-84.

Vogel, J. and K. Papenfort (2006). "Small non-coding RNAs and the bacterial outer membrane." Curr Opin Microbiol **9**(6): 605-11.

Waters, L. S. and G. Storz (2009). "Regulatory RNAs in bacteria." Cell **136**(4): 615-28.

Wiehlmann, L., A. Munder, T. Adams, M. Juhas, H. Kolmar, P. Salunkhe and B. Tümmler (2007). "Functional genomics of *Pseudomonas aeruginosa* to identify habitat-specific determinants of pathogenicity." Int J Med Microbiol **297**(7-8): 615-23.

Wilderman, P. J., N. A. Sowa, D. J. FitzGerald, P. C. FitzGerald, S. Gottesman, U. A. Ochsner and M. L. Vasil (2004). "Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis." Proc Natl Acad Sci U S A **101**(26): 9792-7.

Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser and M. Cashel (1991). "Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations." J Biol Chem **266**(9): 5980-90.

Yan, Y., J. Yang, Y. Dou, M. Chen, S. Ping, J. Peng, W. Lu, W. Zhang, Z. Yao, H. Li, W. Liu, S. He, L. Geng, X. Zhang, F. Yang, H. Yu, Y. Zhan, D. Li, Z. Lin, Y. Wang, C. Elmerich, M. Lin and Q. Jin (2008). "Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501." Proc Natl Acad Sci U S A **105**(21): 7564-9.

Zuker, M. (2003). "Mfold web server for nucleic acid folding and hybridization prediction." Nucleic Acids Res **31**(13): 3406-15.

TABLE 1. Bacterial strains and plasmids

Strains/plasmid	Genotype/relevant features	Source/reference
P. aeruginosa		
PAO1	wild-type	(Holloway *et al.*, 1979)
PAO1*ΔpaeII*	*paeII* in frame deletion mutant	this study
NB159	PAO1Δ*relA*	(Boes *et al.*, 2008)
NB170	PAO1Δ*relA* Δ*spoT*	(Boes *et al.*, 2008)
PAO1 *rpoS*-	PAO1*rpoS101*::*aacCI*, Gm^R	(Suh et al., 1999)
PAO6358	PAO1Δ*rpoN*	(Heurlier et al., 2003)
PAO1*hfq*-	PAO1*hfq*::*aadA*, Sm/Sp^R	(Sonnleitner *et al.*, 2003)
E. coli		
Top10	F-, *mcrA*, Δ(*mrr-hsd*RMS-*mcr*BC),p80*lacZ*ΔM15 Δ*lacX*74, *deoR*, *recA1*, *ara*D139Δ(*ara-leu*)7697, *galK*, *rpsL*(StrR), *endA1*, *nupG*	Stratagene
S17-1	*recA1 pro thi*; the *tra* genes of plasmid RP4 are integrated in the chromosome	(Simon *et al.*, 1986)
plasmids		
pGEM-T	A/T cloning vector, Ap^R	Promega
$pHFQ_{Pa}$	expression vector for purification of PAO1 Hfq	(Nikulin *et al.*, 2005)
pME4510	Broad-host-range promoter-probe plasmid, Gm^R	(Rist and Kertesz, 1998)
pMEPaeII	pME4510 with *paeII* promoter and *paeII* gene	this study
pMEPaeII-RpoN5	pMEPaeII with mutated σ^{54} promoter	this study
pSUP202	pBR325, Ap^R, Cm^R, Tc^R, *mob*	(Simon *et al.*, 1983)
pSUP202-PaeIIdn	pSUP202 with *paeII* downstream fragment	this study
pSUP202-PaeIIko	pSUP202 with *paeII* deletion	this study

TABLE 2. Oligonucleotides used in this study

Name	Sequence[a]	Binding region[b]
I_26	CCCCACACTACCATCGGCGATGCGTCG	6039619-6039645
V_28	GGCTTTTCTGGGGTGGGGTTGGCTT	4523929- 4523905
Q_53	TTTT**CGATCG**ACCTGCCATTCGAACAGTTG	4523284-4523303
R_53	TTTT**GAATTC**TACTCTAGCAAACTCGGCCA	4523836-4523817
S_53	TTTT**GAATTC**CAGAAAAGCCGGCCACAA	4523920-4523937
T_53	TTTT**CCATGG**GACGCGATGAACCGCTAC	4524521-4524504
V_51	GTGGGGTTGGCTTACTTCT	4523917-4523900
W_51	CAGAAAAGCCGGCCACAA	4523920- 4523937
K_29	GC**TCTAGA**GCGGGATGCTGGGCAGGACTGG	4523798-4523819
L_29	G**GAATTC**GCATTAAAAAAGCCGGCTTGTGG	4523954-4523932
Y_57	TTT**GAATTC**GCGGGATGCTGGGCAGGAC*AC*GCCGAGTT*AC*ATAGAGTAGCGCTTC	4523798-4523843
A_57	TGC**TCTAGA**CGTAATACGACTCACTATAGTCCTTCACGAGCCCTCGGC	4523842-4523860
U_26	CCG**TCTAGA**CGTAATACGACTCACTATAGTCAGGACATTGCGCAGGAAGC	586867-586888
V_26	AAA**CTGCAG**GAGCGACGCGGTTTTCCTCGGGC	587018-586996
L_37	ATCGTAGTCCGGATCGCAGT	723383-723402
M_37	AGTCATGAATCACTCCGTGGTA	723574-723553
O_45	TTTTTT**GGATCC**CATGGCGCGTGAGCTTACCAC	4455889-4455869
N_45	TTTTTT**CTGCAG**CGACCTGCTCGTTGAAGCTCACCGC	4455601-4455625
E_2	CTTCTTCCGACTGGCTGCGGG	5308717-5308737

a. Restriction sites are highlighted in bold, T7 promoter sequences are underlined and introduced mutations are in italics.

b. Binding position to PAO1 genome based on the *Pseudomonas aeruginosa* PAO1 genome database: http://v2.pseudomonas.com.

TABLE 3. Microarray data

PAO1 versus PAO1Δ*paeII*

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

a. Gene numbers are taken from the *Pseudomonas* genome project (http://www.pseudomonas.com).
b. The change means difference of the respective transcript levels in strain PAO1 when compared to PAO1Δ*paeII*. –downregulation; + upregulation.
c. RND: Resistance-Nodulation-Cell Division.

Figure Legends

Fig. 1. Predicted PaeII secondary structure, PaeII conservation and expression signals. **(A)** Secondary structure prediction of PaeII using the program mfold (Zuker, 2003). **(B)** Northern-blot analysis of total RNA from PAO1 cells grown in LB to OD_{600} of 1 using a strand-specific probe for PaeII (see Materials and Methods). The size of the marker (M) is indicated at the left. Arrows mark the full-lenght (F: 108 nt) and the processed (P: 76 nt) form of PaeII. **(C)** Sequences of putative *paeII* promoters. The predicted σ^{70} promoter region is underlined and the probable RpoN-dependent promoter is boxed. Mutations introduced in the putative RpoN promoter sequence are indicated. The transcriptional start site is underlined and in italics. The 5' and 3' ends mapped by 5'-3' circularisation are indicated by black arrows. The open arrow marks the processing site, which has been mapped by the 5'-3' circularisation procedure without TAP-treatment of total RNA. The recognition sequence of oligonucleotide V_28, which has been used for Northern-blot analysis, is indicated bold. **(D)** Sequence alignment of PaeII in pseudomnads using BLASTN 2.2.23+ (Altschul *et al.*, 1997). (*P.a.*: *P. aeruginosa* PAO1/PA14/PA7/LESB57, *P.m.: P. mendocina, P.f.: P. fluorescens, P.e:P. entomophila, P.s.: P. stutzeri, P.p.: P. putida*)

Fig. 2. Mapping of RNase E cleavage sites in PaeII RNA. 5′-[^{32}P]-labelled PaeII RNA (0.15 pmol) was incubated in the absence (lanes 4 and 5) or in the presence (lanes 6-9) of Hfq_6. The molar ratios of protein:RNA are indicated on top of the autoradiograph. *In vitro* RNase E cleavage of PaeII RNA was performed in the absence (lane 5) or in the presence (lanes 6 and 7) of Hfq_6. The arrows on the right numbered 1 and 2 at the right indicate RNase E cleavage sites in PaeII and the arrows 1 and 2 on the left denote the RNaseE cleavage sites in the *paeII* sequence (from position +1 to +51). In lane 1, 2 and 3 an OH, RNase A and RNase T1 ladder was loaded, respectively.

Fig. 3. Nutrient limitation stimulates *paeII* expression. **(A)** PaeII levels were assessed by Northern-blot analysis of total RNA from PAO1 grown under different conditions (lane 1: LB OD_{600}=1, lane 2: BM2 minimal medium OD_{600}=1, lane 3: LB OD_{600}=3, lane 4: oxidative stress, lane 5: osmotic stress, lane 6: heat stress, lane 7: cold stress, lane 8: iron depletion and lane 9: low pH). For PaeII the probe V_28 was used and as an internal control probe I_26 for 5S rRNA. **(B)** The PaeII steady state levels in BM2 minimal medium supplemented with 20 mM succinate at different cell densities (OD_{600}= 0.5, 1, 1.8 and 2.6) were determined by Northern-blot analysis. The experiment was done in duplicate. **(C)** The influence of different C-sources (succinate, mannitol, glycerol and glutamate, glucose) and of the salt composition (BM2 minimal medium and M9 minimal medium) on *paeII* expression was assessed by Northern-blot analysis as specified in (A). The experiment was done in duplicate.

Fig. 4. Transcriptional regulation of *paeII*. **(A)** The steady-state levels of PaeII in PAO1 (lane 1) and in strain PAO1Δ*rpoN* (lane 2) were determined by Northern-blot analysis (see Fig. 3A) upon growth in BM2 minimal medium supplemented with 20 mM mannitol and 20 mM glutamine at an OD_{600}=1.0. The PAO1Δ*rpoN* mutant strain is auxotroph for glutamine (Totten *et al.*, 1990). Therefore the minimal medium for PAO1 and PAO1Δ*rpoN* was supplemented with 20 mM glutamine. **(B)** Determination of the PaeII steady state levels by Northern-blot analysis from total RNA of PAO1Δ*paeII* carrying plasmid pME4510 (lane 1), pME-PaeII (lane 2, wt promoter) or pME-PaeII-RpoN5 (lane 3, σ^{54} promoter mutations (Fig. 1C)) at an OD_{600} of 1.0 upon growth in BM2 minimal medium supplemented with 20 mM mannitol and 20 mM glutamine. The signals were quantified and normalized to 5S rRNA signals. **(C)** The steady-state levels of PaeII and CrcZ in PAO1 (lane 1), PAO1Δ*relA*Δ*spoT* (lane 2) and PAO1Δ*relA* (lane 3) at an OD_{600} of 1.0 were assessed by Northern-blot analysis (see Fig. 3A) upon growth in BM2 minimal medium supplemented with 20 mM mannitol. The probe V_28 and the probe E_2 were used to detect

PaeII and CrcZ, respectively. Probe I_26 served for detection of 5S rRNA. The transcript levels of PaeII (black bars) and CrcZ (white bars) present in strain PAO1 were set to 100%. The experiment was done in duplicate.

Fig. 5. Hfq influences the PaeII steady state levels. **(A)** PaeII levels remaining upon growth in BM2 minamal medium supplemented with 20 mM mannitol in the presence and absence of Hfq, 0, 2.5, 5, 10 and 20 min after addition of rifampicin, was assessed by Northern-blot analysis (see Fig. 3A). **(B)** Steady state levels of PaeII at an OD_{600} of 1.0 in PAO1 (black bar) and PAO1*hfq*$^{-}$ (white bar). The Northern-blot signals were quantified and normalized to the 5S rRNA signals. The signal obtained for PAO1 was set to 1. The experiment was done in duplicate. **(C)** The relative amount of PaeII at indicated time points in the presence (□) and absence of Hfq (♦) after rifampicin treatment were normalized to that of 5S rRNA. The experiment was done in duplicate.

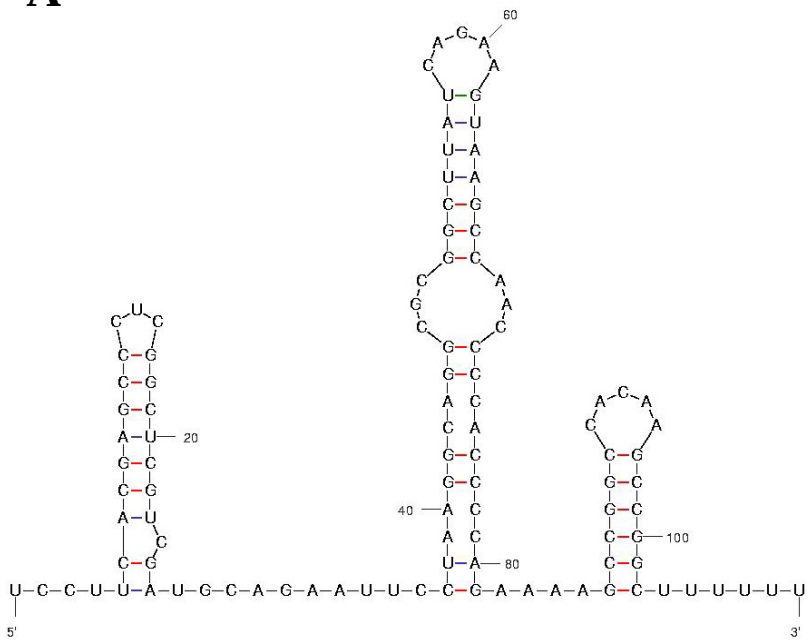
Fig. 6**.** Identification of putative PaeII targets by proteomics. The protein pattern of PAO1 (left panel) was compared to that of PAO1Δ*paeII* (right panel). The protein samples were withdrawn at an OD_{600} of 1.0 after growth in BM2 minimal medium supplemented with 20 mM mannitol. Selected spots with higher (dotted circles 1 and 3) or lower intensity (circles 2 and 4) in the presence of the sRNA PaeII were identified by mass-spectrometry. Putative targets of PaeII were identified as PA1244 (dotted circle 1), PA1579 (circle 2), MvaT (dotted circle 3) and PA3332 (circle 4). Only the relevant section of the gel is shown.

Supplementary Fig. S1. Interaction of Hfq with PaeII. Labelled RNA (5 nM) was incubated in the absence (lane 1) or in the presence of 10 nM (lane 2), 20 nM (lane 3), 40 nM (lane 4), 80 nM (lane 5) or 160 nM (lane 6) hexameric Hfq (Hfq_6). The Hfq_6:RNA ratio is indicated. Unlabelled PaeII (lanes 7 and 8), PAO1 RsmY RNA (lanes 9 and 10) and tRNA (lanes 11 and

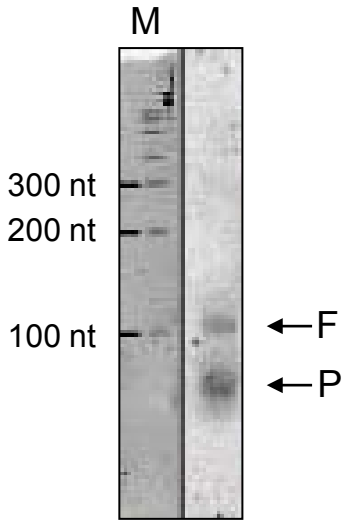
12) were added as competitors. Position of free (F1 and F2) and Hfq-bound (B) RNA are indicated.

Figure 1

A



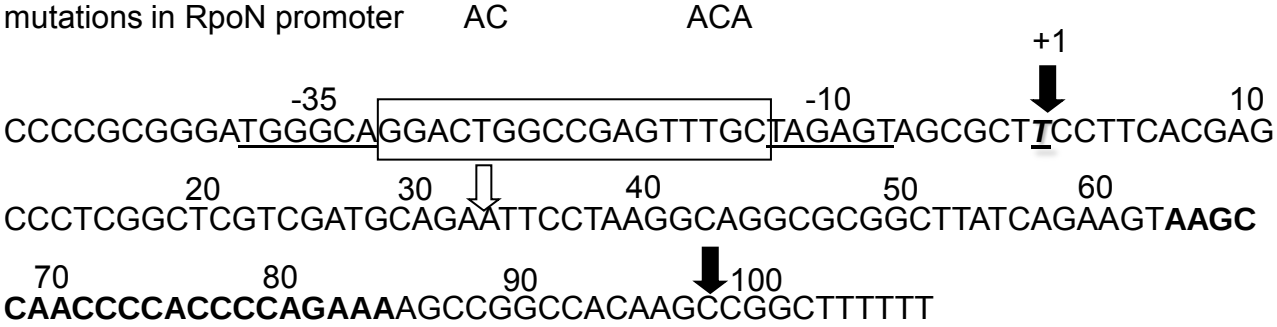
B



C

RpoN consensus YTGGCACGNNNNTTGCWNN

mutations in RpoN promoter AC ACA



D

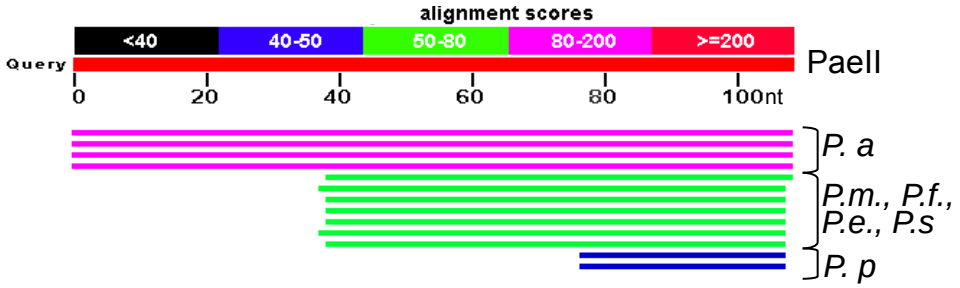


Figure 2

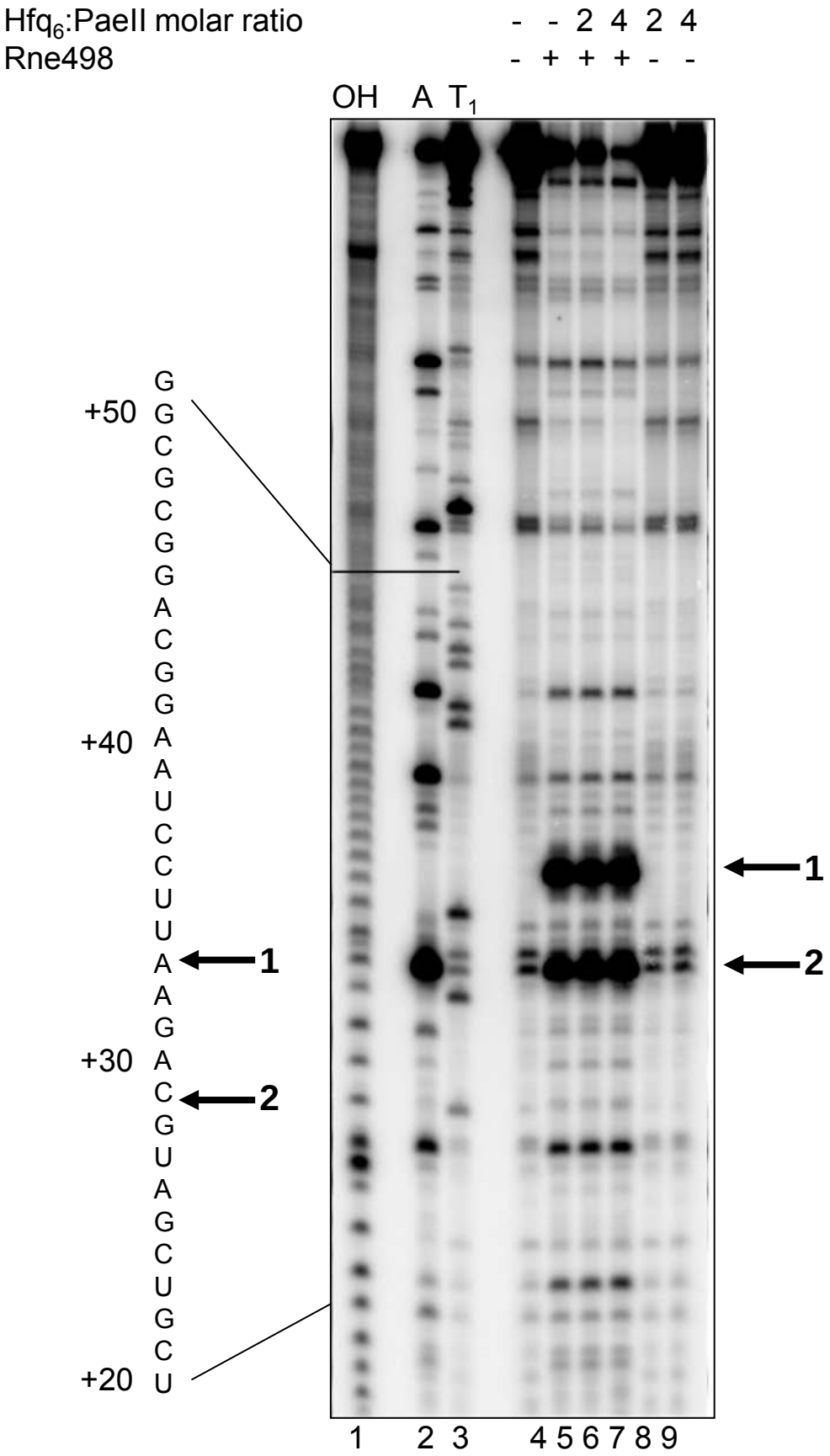

Hfq6:PaeII molar ratio - - 2 4 2 4
Rne498 - + + + - -
OH A T1
+50
+40
+30
+20
G G C G C G G A C G G A A U C C U U A A G A C G U A G C U G C U
1
2
1
2
1 2 3 4 5 6 7 8 9

Figure 3

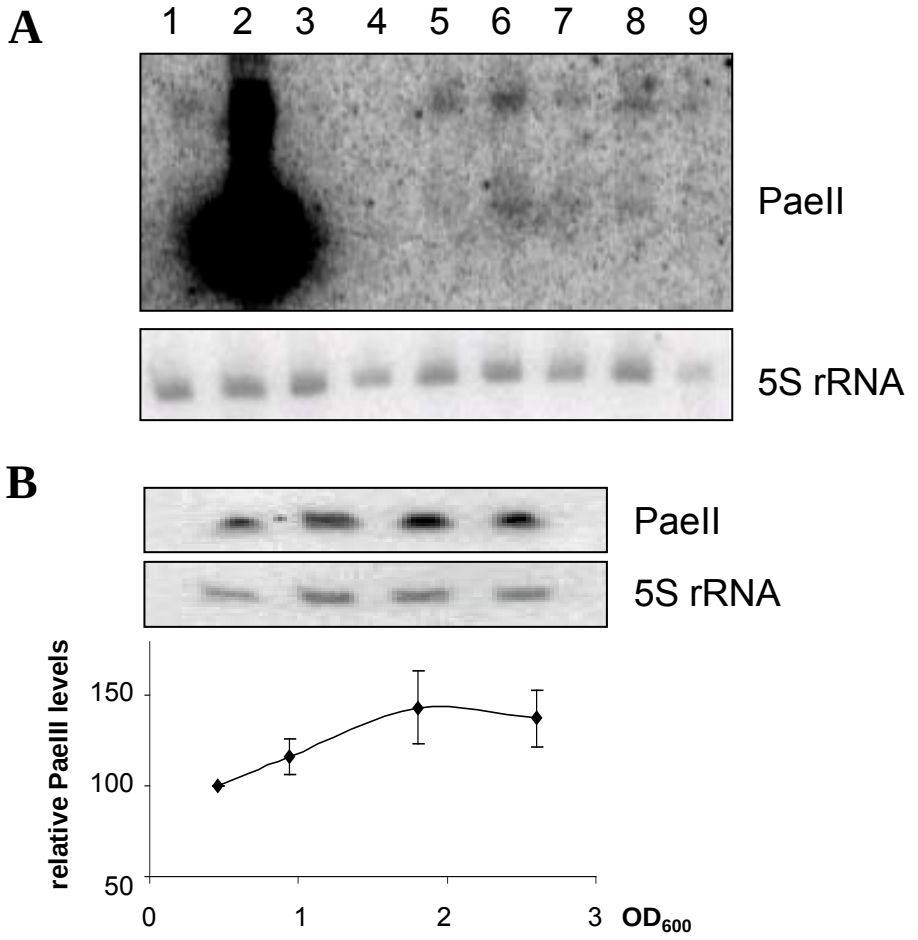

A
1 2 3 4 5 6 7 8 9
PaeII
5S rRNA
B
PaeII
5S rRNA
relative PaeIII levels
150
100
50
0
1
2
3
OD600
C
PaeII
5S rRNA
relative PaeIII levels
2
1,5
1
0,5
0
BM2+succinate
BM2+mannitol
BM2+glycerol glutamate
BM2+glucose
M9+glucose
LB

Figure 4

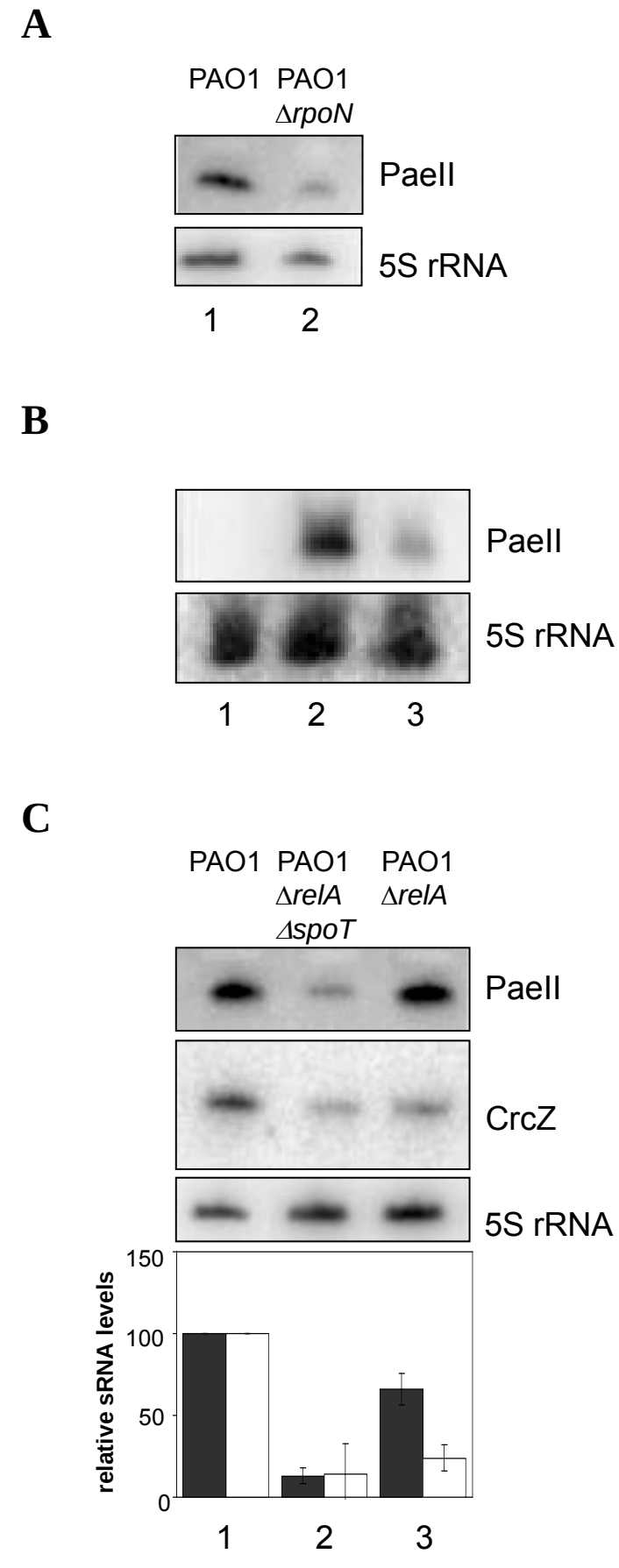
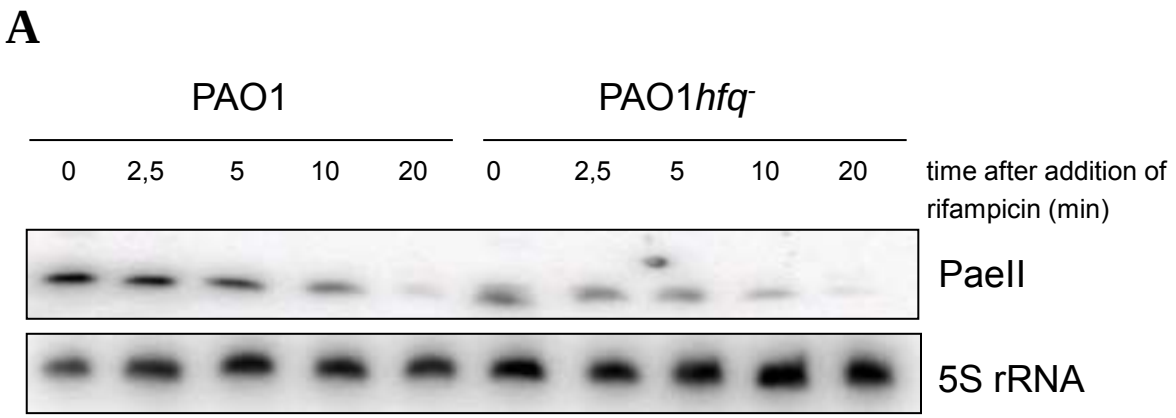
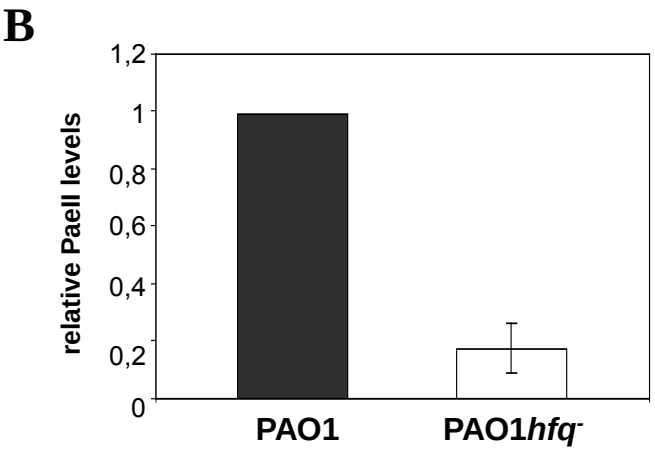

A
PAO1
PAO1
ΔrpoN
PaeII
5S rRNA
1
2
B
PaeII
5S rRNA
1
2
3
C
PAO1
PAO1
ΔrelA
ΔspoT
PAO1
ΔrelA
PaeII
CrcZ
5S rRNA
relative sRNA levels
150
100
50
0
1
2
3

Figure 5


A
PAO1
PAO1hfq-
0 2,5 5 10 20 0 2,5 5 10 20
time after addition of rifampicin (min)
PaeII
5S rRNA


B
relative PaeII levels
1,2
1
0,8
0,6
0,4
0,2
0
PAO1
PAO1hfq-

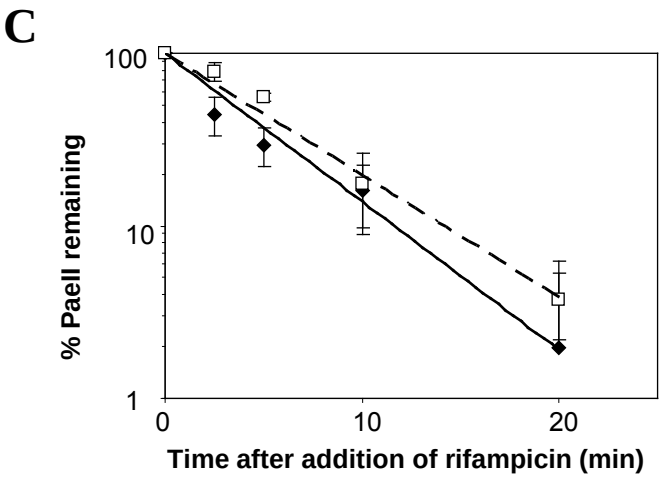
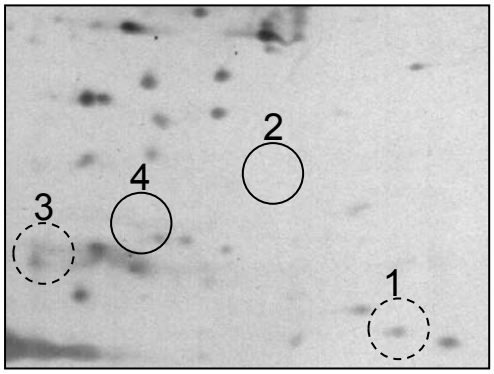
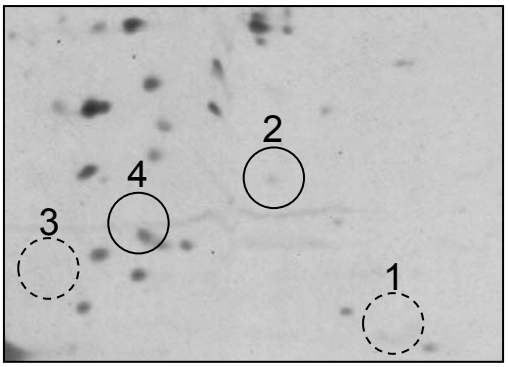

C
% PaeII remaining
100
10
1
0
10
20
Time after addition of rifampicin (min)

Figure 6

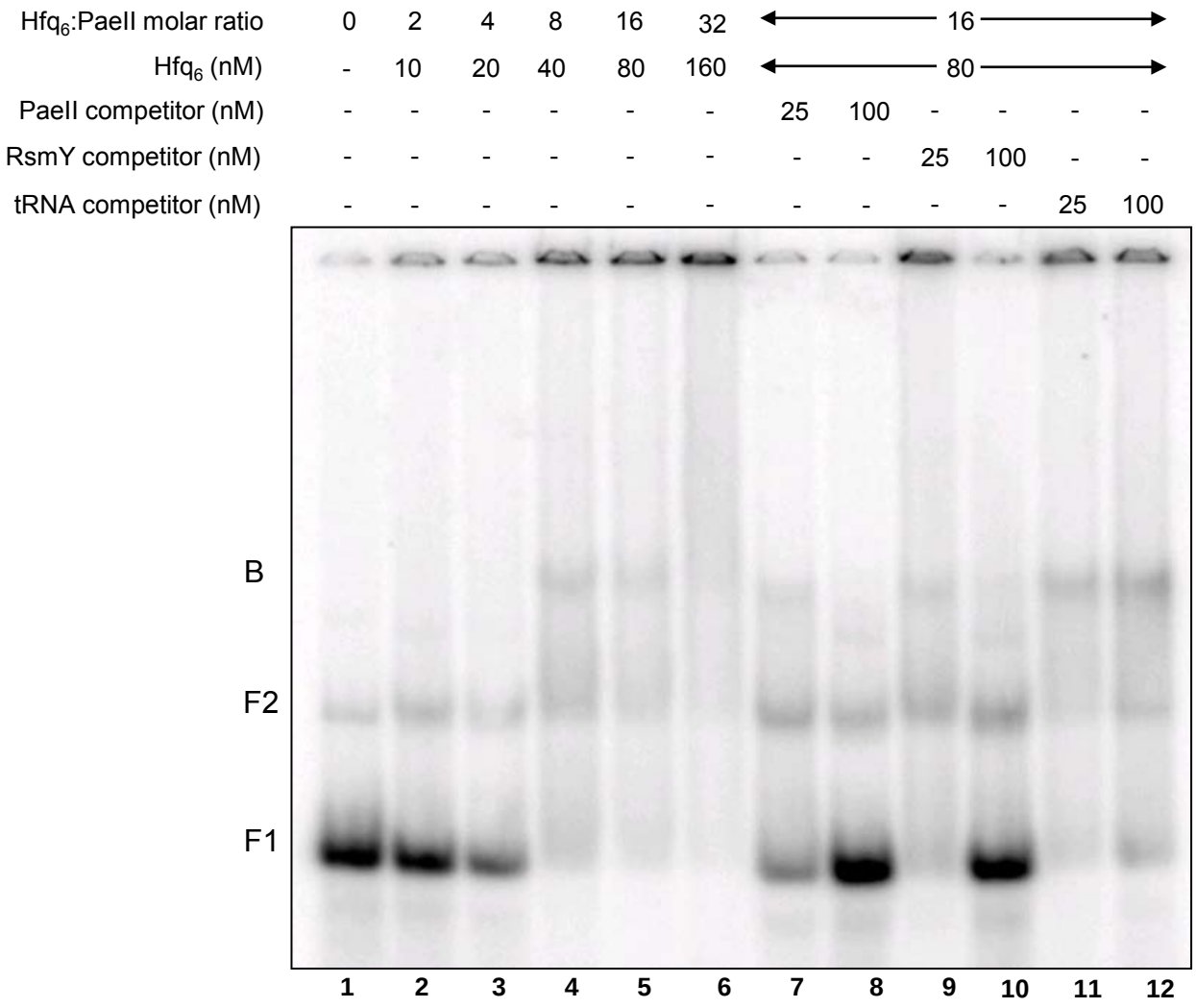
PAO1



PAO1Δ*paeII*



Supplementary Figure S1



4.3 The non-coding RNA PaeIII of *Pseudomonas aeruginosa* affects antibiotic susceptibility and *rpoS* expression

Abstract

Small regulatory RNAs (sRNAs) are known to control various cellular processes including virulence gene expression in several bacterial pathogens. A bioinformatic approach employed to analyze intergenic regions of *Pseudomonas aeruginosa* for sRNAs revealed PaeIII. PaeIII expression is shown to be induced upon entry into stationary phase and in an *rsmA* mutant strain. The proteome and transcriptome of the parental and the *P. aeruginosa paeIII* over-expressing strain were compared to unravel PaeIII targets. The transcriptome analysis revealed that the expression of genes encoding components of the MexXY multidrug efflux system as well as that of genes encoding the Type III secretion apparatus increased, whereas expression of the stationary phase sigma-factor gene *rpoS* as well as expression of genes involved in pyocin production and anaerobic respiration were found to be decreased.

Introduction

P. aeruginosa (PAO1) can adapt to a variety of environments, soil, water as well as to different hosts. As an opportunistic pathogen it can cause severe infections especially in immunocompromised patients. Due to its ability to form biofilms, PAO1 is able to escape host defence mechanisms and antibiotic treatment (Costerton *et al.*, 1999). Many virulence factors of PAO1 are controlled by three interlinked quorum sensing (QS) systems termed Las, Rhl and PQS, numerous two component systems as well as extra-cytoplasmatic sigma factors (for review van Delden and Iglewski, 1998).

Small regulatory RNAs (sRNAs) can modulate stress responses (i) by regulating the expression of the alternative sigma factor RpoS (Repoila *et al.*, 2003), (ii) through their function in iron metabolism (Massé *et al.*, 2007) and catabolite repression (Sonnleitner *et al.*, 2009) or (iii) by remodelling the outer membrane (Vogel and Papenfort, 2006). A large number of sRNAs associate with the RNA chaperone Hfq and require it to exert their function in post-transcriptional regulation (for review Valentin-Hansen *et al.*, 2004; Aiba, 2007; Brennan and Link, 2007; Waters and Storz, 2009). A PAO1*hfq*$^{-}$ mutant was attenuated in virulence, suggesting that sRNAs are involved in pathogenicity (Sonnleitner *et al.*, 2003). To date, the function of only a few PAO1 sRNAs has been studied in greater detail. Prrf1/2 are involved in maintaining iron homeostasis (Wilderman *et al.*, 2004; Oglesby *et al.*, 2008), whereas the GacA controlled RgsA appears to be involved in protection of PAO1 from oxidative stress (Gonzalez *et al.*, 2008). RsmY/Z and CrcZ sequester the translational repressor of virulence genes RsmA (Burrowes *et al.*, 2006; Kay *et al.*, 2006) and the translational repressor Crc involved in catabolite repression (Sonnleitner *et al.*, 2009), respectively. Moreover, approximately 30 sRNAs with unknown function have been recognized in PAO1, most of which have been predicted by biocomputation (Livny *et al.*, 2006; Gonzalez *et al.*, 2008; Sonnleitner *et al.*, 2008), while others were identified by RNomics (Sonnleitner *et al.*, 2008).

Here, using bioinformatics we identified, among other sRNA candidates the novel sRNA PaeIII. Upon verification of *paeIII* expression, we studied conditions that provoke PaeIII synthesis as well as regulatory factors involved. Proteome and transcriptome analyses were performed to reveal potential target genes of PaeIII.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. Unless indicated otherwise, bacterial cultures were grown at 37°C in Luria-Bertani (LB) medium (Miller, 1972), supplemented with appropriate antibiotics. Antibiotics were added to a final concentration of 200 µg/ml spectinomycin-streptomycin, 50 µg/ml gentamicin, 1mg/ml kanamycin, 125 µg/ml tetracyclin and 300 µg/ml carbenicillin for *P. aeruginosa* and 50 µg/ml gentamicin, 50 µg/ml kanamycin, 50 µg/ml tetracyclin and 100 µg/ml ampicillin for *E. coli*.

Bioinformatics

A search algorithm that follows the general scheme described by Lenz *et al.* (2004) was used to unravel novel sRNAs: (i) Most sRNAs are located in intergenic regions, therefore we restricted our search to regions between annotated genes. The *P. aeruginosa* intergenic regions were downloaded from http://www.pseudomonas.com (Intergenic DNA, *Pseudomonas aeruginosa* PAO1, version 2004-01-14). (ii) We assumed that the putative RNAs contain a Rho-independent terminator. 524 *P. aeruginosa* Rho-independent terminators were downloaded from http://www.tigr.org/software/transterm.html, of which 502 were found to be located in intergenic regions. (iii) The upstream region of the RNA loci were searched for a σ^{54}- (or a σ^{70}) binding site. Using PATSER (http://liv.bmc.uu.se:16080/rsa-tools, Pattern Matching, PATSER), the *P .aeruginosa* intergenic regions were screened for potential σ^{54}-binding sites with a weight matrix constructed from a compiled set of ~180 σ^{54}-binding sites from multiple bacterial species (Dombrecht *et al.*, 2002). All matches with a score higher than or equal to the cutoff score 8.9 (Dombrecht *et al.*, 2002) were considered as potential hits. The number of these 214 potential hits was further reduced to 168 by the assumption that the distance between the potential sRNAs and the next coding region should be at least 50 nucleotides. Combining

these 168 hits with the list of Rho-independent terminators resulted in a list of 22 intergenic regions which are supposed to contain both a Rho-independent terminator and a σ^{54}-binding site. A final check of these 22 hits (the σ^{54}-binding site and the terminator sequences must have the same orientation - plus or minus, length and localization of the potential sRNAs within the intergenic region) left us with one sRNA candidate located in the intergenic region between the ORFs PA1781-PA1782.

A similar procedure was carried out to detect potential sRNAs carrying both a σ^{70}-binding site and a Rho-independent terminator. Again, the *P. aeruginosa* intergenic regions were screened with PATSER. We tested all of the best 11 weight matrices for the σ^{70}-binding site recognition (Huerta and Collado-Vides, 2003). The results were combined with the list of Rho-independent terminators and length and distance restrictions were applied. As a result of this procedure, we obtained four potential sRNA candidates, two of them are located in the intergenic region between the ORFs PA4704-PA4705, named PrrF1 and PrrF2 (Wilderman *et al.*, 2004), and the candidates in the intergenic region between the ORFs PA3535-PA3536 and the intergenic region between the ORFs PA4040-PA4041.

Construction of a *paeIII* deletion mutant

The sequence upstream (nucleotides -655 to -26 with regard to the transcriptional start) and the downstream sequence (nucleotides +62 to +666) of *paeIII* were amplified by PCR using chromosomal PAO1 DNA as a template and the oligonucleotides V_34 and W_34 as well as Q_32 and R_32 respectively (Table 2). The 629 nt long upstream fragment was cloned into the *PstI* and *PvuI* site of plasmid pSUP202, resulting in pSUP202-PaeIIIup. Thereafter, the 604 nt long downstream sequence, containing the *paeIII* Rho-independent terminator sequence, was cloned into the *PvuI* and *EcoRI* site of pSUP202-PaeIIIup, resulting in pSUP202-PaeIIIko. This plasmid was transformed into the *E. coli* strain S17-1 and transferred by conjugation to PAO1.

The following homologous recombination resulted in the chromosomal deletion of *paeIII*. The deletion was verified by PCR and Northern-blot analysis.

Mapping of the 5´and 3´termini of PaeIII

Simultanous mapping of the 5' and 3' ends of PaeIII was performed as previously described (Toledo-Arana *et al.*, 2009). 6 µg of total RNA was incubated for 45 min at 37ºC with the corresponding buffer, in the presence or absence of Tobacco Acid Pyrophosphatase (TAP) (Epicentre biotechnologies). This step allows to distinguish between a 5'-end generated by transcription initiation and a 5'-end resulting from endonucleolytic processing. After phenol and chloroform extraction and ethanol precipitation, serial dilutions (from 500 ng to 0.5 ng) of the TAP+ and TAP- treated RNAs were prepared. Each sample was ligated with 40 U of T4 RNA ligase I (Fermentas) in the presence of 1x RNA ligase buffer supplemented with 8% DMSO, 10 U of RNase Inhibitor, 1 U of DNase I and RNase-free water in a total volume of 25 µl at 37ºC for 1 h. After phenol and chloroform extraction and ethanol precipitation, the ligated RNAs were resuspended in 10 µl of RNase-free water. The RT-PCR reactions were performed using specific outward primers B_53 and C_53 (Table 2) and the SuperScript One-Step RTPCR kit (Invitrogen). Bands only present in the TAP+ reactions with the expected size were purified using the Gel Extraction kit (QIAGEN) and cloned using the pGEM®-T Easy Vector System (Promega). 10 plasmid clones containing the expected size of insert were sequenced. The sequences were compared with the *P. aeruginosa* genome (www.pseudomonas.com) to localize the 5'- and 3'-ends.

Detection of PaeIII by Northern-blot analysis

Total RNA of PAO1 was purified using the hot phenol method (Lin-Chao and Bremer, 1986). The abundance of the RNAs was determined by Northern-blot analysis using total RNA and the signals were normalized to the hybridization signal obtained for ribosomal 5S rRNA.

The RNA samples were denatured for 5 min at 65°C in loading buffer, containing 50% formamide, separated on 8% polyacrylamide – 8 M urea gels, and then transferred to nylon membranes by electroblotting. The RNAs were crosslinked to the membrane by exposure to UV light. The membranes were hybridized with gene-specific ^{32}P-end-labelled oligonucleotides W_28 (PaeIII) and I_26 (5S rRNA) (Table 2) over night and the hybridization signals were visualized with a PhosphoImager (Molecular Dynamics).

Construction of a PaeIII harboring plasmids

To over-express PaeIII under its own promoter plasmid pME-PaeIII was constructed. The DNA sequence of *paeIII*, including its authentic promoter was amplified by primer pairs N_29 and O_29 (Table 2). The fragment was blunt-end ligated into the *SmaI* site of vector pME4510. PaeIII over-expression was verified by Northern-blot analysis (Supplementary Fig. S3).

To construct a plasmid bearing an inducible copy of *paeIII*, the full lenght *paeIII* sequence was amplified by PCR using primers V_40 and J_40 (Table 2), which contains a *SpeI* and a *KpnI* restriction site, respectively. After cleavage with these enzymes the 107 nt long fragment was cloned into the *SpeI* and *KpnI* restriction sites of plasmid pJT19, resulting in pJT19-PaeIII. The *paeIII* over-expression was verified by Northern-blot analysis (Fig. 5A).

Construction of reporter-gene fusions

The *tac* promoter of pME3843 (Blumer *et al.*, 1999) was PCR-amplified using oligonucleotides Z_34, which creates a *EcoRI* site, and primer A_35, which creates a *BamHI* site (Table 2). The 930 bp fragment containing the *tac* promoter was inserted into the *EcoRI* and *BamHI* sites of plasmid pME3846 (Pessi and Haas, 2001). To construct a *purE* translational fusion to *lacZ*, transcription of which is driven by the *tac* promoter, the *purE* fragment was amplified by PCR using oligonucleotides J_39 and K_39 (Table 2) from

chromosomal PAO1 DNA. The 97 nt long fragment was cleaved with *BamHI* and *PstI* and cloned into the corresponding restriction sites of plasmid pME*tac*, resulting in pME*tac*-PurE.

To construct a translational fusion between PAO1 ORF PA1202 and *lacZ* the PA1202 fragment was amplified by PCR using oligonucleotides B_35 and C_35 (Table 2). Upon cleavage with *PstI* and *BamHI* the 127 nt long fragment was cloned into the *BamHI* and *PstI* sites of plasmid pME*tac*, resulting in pME*tac*-PA1202.

For transcriptional and translational fusions of *rpoS*, *mexZ* and PA5471 to *lacZ* the plasmids pME6016 and pME6014 were used. In contrast to the aforedescribed translational *lacZ* fusions, here the respective gene was under transcriptional control of the authentic promoter. Therefore, the readouts of the translational *lacZ*-fusions (pME6014) reflect transcriptional and translational changes. To distinguish this effects on transcription from that on translation, transcriptional fusions were as well constructed using plasmid pME6016 (Table 1).

To construct a *rpoS-lacZ* fusion gene a PCR fragment containing the *rpoS* promoter and the first 56 codons was amplified by PCR using the oligonucleotides A_52 and B_52 (Table 2), which contain a *BamHI* and a *PstI* restriction site, respectively. The 755 nt long fragment was cloned into the *BamHI* and *PstI* restriction sites of plasmids pME6014 and pME6016, resulting in plasmids pME6014-RpoS (translational *lacZ*-fusion) and pME6016-RpoS (transcriptional *lacZ*-fusion), respectively.

For construction of the *mexZ-lacZ* fusion a PCR fragment containing the *mexZ* promoter and the first 7 codons was amplified by PCR using the oligonucleotides T_47 and U_47 (Table 2), which contain an *EcoRI* and a *PstI* restriction site, respectively. The 195 nt long fragment was cloned into the *EcoRI* and *PstI* restriction sites of plasmids pME6014 and pME6016, resulting in plasmids pME6014-MexZ (translational *lacZ*-fusion) and pME6016-MexZ (transcriptional *lacZ*-fusion), respectively.

For construction of the ORF PA5471-*lacZ* fusion a PCR fragment containing the PA5471 promoter and the first 9 codons was amplified by PCR using oligonucleotides V_47 and W_47 (Table 2), which contain an *EcoRI* and a *PstI* restriction site, respectively. The 406 nt long fragment was cloned into the *EcoRI* and *PstI* restriction sites of plasmids pME6014 and pME6016, resulting in plasmids pME6014-PA5471 (translational *lacZ*-fusion) and pME6016-PA5471 (transcriptional *lacZ*-fusion), respectively.

Expression of *paeIII* under different stress conditions

To test *paeIII* expression under different stress conditions, strain PAO1 was grown in LB at 37°C to an OD_{600} of 1.0 and subsequently exposed to 43°C for 15 min (heat shock), to 15°C for 30 min (cold shock), to 1mM hydrogen-peroxide for 15 min (oxidative stress), to 500 µM Dipyridyl for 15 min (iron deprivation), to 0.3 M NaCl for 15 min (osmotic shock) or to HCl, pH=3 for 15 min (low pH). To mimic starvation PAO1 was grown in BM2 minimal medium (40 mM K_2HPO_4, 22 mM KH_2PO_4, 7 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 0.01 mM $FeSO_4$) supplemented with 20 mM succinate to an OD_{600} of 1.0. After these treatments, total RNA was isolated and 10 µg of the total RNA were subjected to Northern-blot analysis, using oligonucleotides W_28 (PaeIII) and I_26 (5S rRNA) (Table 2).

RNA preparation

PaeIII RNA was *in vitro* transcribed with T7 RNA polymerase (Epicentre AmpliScribe™ T7-*Flash*™ Transcription Kit) from PCR templates. The *paeIII* PCR fragment was amplified using the forward primer M_31 and the reverse primer O_29 (Table 2). The forward primer M_31 contained a T7Φ10 promoter sequence (Table 2, underlined). After *in vitro* transcription the sRNA was purified on a 6% polyacrylamide - 8 M urea gel, and the RNA concentration was determined by measuring the absorbance at 260 nm.

Hfq binding assay

Gel purified PaeIII RNA was 5'-end labelled with [γ-^{32}P]-ATP (Amersham), and re-purified on a 6% polyacrylamide-8M urea gel as described (Sonnleitner *et al.*, 2006). Labelled RNA (0.05 pmol) was first incubated at room temperature for 5 min to refold, then for 15 min with or without increasing concentrations of purified Hfq-hexamer (Hfq_6) (0.1, 0.2, 0.4, 0.8, 0.16 or 0.32 pmol) in 10 µl VD buffer (10 mM Tris, pH 7.4, 60 mM NH_4Cl, 6 mM β-mercaptoethanol and 2 mM Mg-acetate). 0.25 and 1 pmol PaeIII and tRNA were added as competitors to the labelled PaeIII RNA. Immediately before loading, the samples were mixed with 4 µl of loading dye (75% glycerol, 0,01% xylencyanol, 0,01% bromphenol blue) and loaded on a native 4% polyacrylamide gel. Electrophoresis was performed in TAE buffer at 160 V at 4°C. The radioactively labelled bands were visualized with a PhosphoImager (Molecular Dynamics).

Determination of PaeIII stability and abundance

The steady state levels and the stability of PaeIII were determined in strains PAO1 and PAO1*hfq*$^-$. At an OD_{600} of 1, rifampicin (500 µg/µl) was added and 4 ml aliquots were withdrawn 0, 15, 30, 60 and 90 min thereafter for isolation of total RNA. Total RNA was purified by the hot phenol method (Lin-Chao and Bremer, 1986). The PaeIII concentrations were determined by Northern-blot analysis (2,5 µg total RNA) using the 5'-end labeled oligonucleotide W_28 for PaeIII (Table 2). The 5S rRNA was used as an internal control and was detected with the oligonucleotide probe I_26 (Table 2).

Proteomics

To detect PaeIII-mediated regulatory effects, differences in the proteome profile were analyzed, upon plasmid-directed over-expression of the RNA. Total cellular extracts of strain PAO1 (pME4510) and PAO1 (pME-PaeIII) were compared by two-dimensional gel

electrophoresis. The strains were grown in LB to an OD_{600} of 2, and equal amounts of cells were dissolved in lysis buffer (8 M urea, 4% (w/v) CHAPS and 40 mM Tris-base). The cells were disrupted by repeated freezing in liquid N_2 and thawing at 37°C followed by incubation at 37°C for 1 h to achieve complete lysis. For the first dimension, the Immobiline Dry Strip pH 3–10 (18 cm; Amersham Pharmacia Biotech) was used with the following IEF program: 12 h rehydration at 40 V, 0.5 h at 300 V, 0.5 h at 1000 V, 0.5 h at 2000 V, 0.5 h at 3000 V, 9 h at 7000 V at 18°C (IPGphor isoelectric focusing system). Resolution in the second dimension was performed on 12% SDS-polyacryamide gels for 15 min at 15 mA, and then for 4 h at 20 mA. Buffers and conditions were used according to the manufacturer's instructions. The gels were silver-stained as described previously (Shevchenko *et al.*, 1996). Selected protein spots with different intensities were identified and excised from the gel to assess the protein identities by mass spectrometry.

Transcriptomics

PAO1 carrying either the empty backbone vector pJT19 or the *paeIII* over-expression plasmid pJT-PaeIII was grown in LB medium to an OD_{600} of 1.0. Then, *paeIII* expression was induced with 2mM toluate. 30 minutes after induction total RNA treated with RNA protect bacteria reagent® (Qiagen) was purified by using the hot phenol method as described (Leoni *et al.*, 1996) followed by DNase I treatment (Roche). Briefly, the cell pellets of 10 ml cultures were mixed with pre-heated (65°C) 2.5 ml lysis buffer (2% Sodium dodecyl sulfate, 30 mM Na-acetate and 3 mM EDTA, pH 5.5) and 5 ml Phenol (pH 5.5). The mixture was vigorously shaken for 8 min, incubated for 5 min on ice, and then centrifuged at 4°C for 15 min at 5000 rpm. Total RNA was purified by phenol-chloroform extraction and precipitated with ethanol and Na-acetate (pH 5.5). To eliminate DNA contamination, the total RNA was treated with 40 Units of DNAseI (Roche) followed by phenol-chloroform extraction and ethanol precipitation. Successful DNAse treatment was verified by PCR. The integrity of total RNA was confirmed

by agarose gel electrophoresis and an RNA 6000 Nano LabChip in an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). For the microarray analysis *P. aeruginosa* GeneChip arrays (Affymetrix, Inc., Santa Clara, CA) were used. cDNA synthesis, fragmentation, labelling, hybridization, staining, and washing steps were performed according to the manufacturer's protocol for the Affymetrix *P. aeruginosa* GeneChip arrays at the University of Lausanne, Center for Integrative Genomics, as described previously (Frangipani *et al.*, 2008). For each condition, the cultures were grown in triplicate, and RNAs from these cultures were pooled before proceeding to cDNA synthesis. In addition, biological replicates for each condition were performed on a separate day and run on a different microarray chip. The over-expression of *paeIII* under these condition was verified by Northern-blot analysis as described above with ^{32}P-end-labelled oligonucleotides W_28 (PaeIII) and I_26 (5S rRNA) (Table 2).

β-Galactosidase assays

The PAO1 strains were grown in LB under aeration at 37°C, triplicate samples were withdrawn and β-Galactosidase activities were quantified as described elsewhere (Miller, 1972).

Results and Discussion

PaeIII, a 98 nt long PAO1 sRNA

A bioinformatic screen revealed the putative sRNA PaeIII, which is located in an intergenic region located between two hypothetical proteins, PA3535 and PA3536 (Stover *et al.*, 2000) (Fig. 1A). A Northern blot anaylsis verified expression of the *paeIII* gene (Fig 1B). The transcriptional start was mapped to the genome position 3958053 by 5'-3' circularisation (Fig. 1C). The sRNA is predicted to be transcribed from a σ^{70} dependent promoter (Fig. 1C, underlined and in italics). The presence of only one signal for the sRNA in the Northern-blot indicated that the 98 nt long sRNA is not processed (Fig. 1B). According to secondary structure predictions, the sRNA consists of two stem-loops and a strong Rho-independent terminator (Fig. 1D). According to BLASTN analysis (Altschul *et al.*, 1997) the sequence of the PaeIII is only conserved among the *P. aeruginosa* strains PAO1, UCBPP-PA14, LESB58 and PA7, but no homologs were found in other pseudomonads.

PaeIII synthesis is decreased in the presence of Hfq

The RNA chaperone Hfq is known to mediate sRNA-mRNA interactions. In PAO1 Hfq was shown to bind to and to stabilize the sRNA RsmY (Sonnleitner *et al.*, 2006; Sorger-Domenigg *et al.*, 2007), and to bind a number of other sRNAs (Sonnleitner *et al.*, 2008). The steady state levels of PaeIII in strains PAO1 and PAO1*hfq*$^{-}$ were determined by Northern-blot analysis at different growth phases (Fig. 2A). When compared to strain PAO1, the steady state levels of PaeIII were increased in strain PAO1*hfq*$^{-}$ (Fig. 2A and B). The stability of PaeIII was comparable in the presence and absence of Hfq (Fig. 2C). PaeIII is very stable, even 90 minutes after inhibition of transcription, no reduction in the amounts of PaeIII was observed (Fig. 2C). In band-shift assays, PaeIII was observed to bind to Hfq but with a rather low affinity (Kd~100 nM) (see Supplementary Fig. S1), which can explain why PaeIII was not

detected in a RNomics approach upon co-immunoprecipitation with Hfq (Sonnleitner *et al.*, 2008). The higher abundance of PaeIII in the absence of Hfq may be explained by an indirect inhibitory effect of Hfq on transcription of PaeIII.

Expression of *paeIII* is growth-phase and RsmA-dependent

Next, *paeIII* expression was assessed upon exposure of the cells to different stress conditions, including heat-, cold-, osmolarity-, pH-, iron depletion-, stationary phase- and oxidative stress as well as nutrient starvation. The highest levels of PaeIII were detected in stationary phase (Fig. 3A, lane 3) and upon pH stress (Fig. 3A, lane 9). However, *paeIII* expression did not depend on the stationary phase sigma factor RpoS. The PaeIII steady state levels (Fig. 3B, lanes 11 and 12) and the stability of the sRNA (data not shown) were not altered in an *rpoS* mutant strain.

To identify regulators involved in *paeIII* expression, we next assessed PaeIII synthesis in different PAO1 strains deficient for distinct transcriptional regulators. Total RNA from the different PAO1 mutant strains was isolated and analyzed for PaeIII levels by Northern-blot analysis (Fig. 3B). When compared with the wild-type strain (Fig. 3B, lane 2), the expression of *paeIII* was increased in an *rsmA* mutant strain by two-fold in stationary phase (Fig. 3B, lane 16). Thus far, RsmA was only shown to act as a translational repressor but not at the transcriptional level. The protein RsmA blocks translation by binding to GGA motifs in the 5' UTR of target mRNAs (Schubert *et al.*, 2007; Lapouge *et al.*, 2008). In stationary phase the sRNA RsmY alleviates this inhibitory effect by sequestering RsmA (Lapouge *et al.*, 2008). Hence, it seems conceivable that RsmA acts by repressing a positive regulator required for *paeIII* transcription.

In addition, growth in synthetic sputum cystic fibrosis medium (Palmer *et al.*, 2007) (Fig. 3B, lane 3) and the absence of RpoN (Fig. 3B, lane 13) resulted in increased *paeIII* expression. Likewise, exposure of *P. aeruginosa* to human serum (Sonnleitner *et al.*, 2008), -as a means to

mimic the human environment during systemic infections-, appeared to induce *paeIII* expression in the PAO1 (Fig. 3C, lane 2) as well as in the PAO1*hfq*- strain (Fig. 3C, lane 4) in comparison to cells grown in LB (Fig. 3C lanes 1 and 3). These results indicated that PaeIII might play a role during infection, and therefore might be involved in the regulation of virulence gene expression.

Proteome analysis upon over-expression of PaeIII.

Riboregulation by sRNAs can result in decreased or increased synthesis of the protein encoded by the target mRNA. Therefore, we first used a proteome approach as a means to identify PaeIII targets. PaeIII was cloned into plasmid pME4510 under its own promoter. As shown in Supplementary Fig. S2, when compared to PAO1 (pME4510) the gene dosage effect resulted in increased expression of *paeIII* in strain PAO1 (pME-PaeIII). Next PAO1 (pME4510) and PAO1 (pME-PaeIII) were grown in LB medium to an OD_{600} of 2.0. Then, the 2D protein pattern of PAO1 (pME4510) was compared to that of PAO1 (pME-PaeIII), and three distinct protein spots (Fig. 4A and B) were selected for analysis by mass-spectrometry. The protein PA1202 was up-regulated at increased levels of PaeIII (Fig. 4A). PA1202 is annotated as a probable hydrolase with structural features of the isochorismatase family of enzymes. PA1202 is 84% identical to the *E. coli* protein YcaC, of which the crystal structure has been determined (Colovos *et al.*, 1998). However, its function remains unknown. Two proteins that were downregulated upon PaeIII over-expression were identfied as phosphoribosylaminoimidazole carboxylase PurE and PA4739, which is 60% identical to the inducible periplasmic protein OsmY from *E. coli* (Fig. 4B). PA4739 is located in an operon together with PA4738 and the corresponding protein is exported (Lewenza *et al.*, 2005). PurE encodes the catalytic subunit of the enzyme phosphoribosylaminoimidazole carboxylase that is involved in purine biosynthesis. The *purE* gene is situated in an operon together with *purK*,

encoding the other subunit of the enzyme. No hints for a concerted regulation of PA1202, PA4738 and PurE were found in the literature.

Next, we tested whether PaeIII acts as a riboregulator on the mRNAs encoding these identified proteins. When compared to PAO1 (pME4510) no differences in the translational efficiency conferred by both, the PA1202-*lacZ* and the *purE-lacZ* fusion, in the *paeIII* over-expressing strain PAO1 (pME-PaeIII) were observed (Supplementary Fig. S3A and S3B). Furthermore, *in vitro* translation of PA1202 was not affected by the presence of *paeIII* RNA (data not shown). Most likely the positive effect of PaeIII on the synthesis of both proteins is indirect.

Microarray analysis reveals PaeIII targets.

Microarrays are a useful tool to monitor changes in RNA levels at a genome wide scale. Duplex formation between a sRNAs and its target mRNAs can lead to degradation, translational inhibition or stabilization of the mRNA (Vogel and Wagner, 2007). Strain PAO1 (pJT19) and strain PAO1 (pJT-PaeIII) were grown in LB to an OD_{600} of 1.0. Then, toluic acid was added to induce transcription of *paeIII*, and 30 min afterwards samples were withdrawn. Under these conditions the control strain expressed moderate levels of *paeIII*, whereas a significant increase was observed in the *paeIII* over-expressing strain (Fig. 5B). PaeIII over-expression resulted amongst others in increased levels of genes encoding the type III secretion system (T3SS) and secreted factors (Hauser, 2009) (Fig. 5B and Table 3). The *P. aeruginosa* T3SS is induced *via* ExsA upon host contact and low extracellular calcium concentrations, whereas it is repressed by metabolic stress, DNA damage, high concentrations of extracellular copper and low osmolarity (Yahr and Wolfgang, 2006). However, only sub-populations respond to these signals (Hornef et al., 2000; Rietsch and Mekalanos, 2006), leading to a phenomenon called bistability (Dubnau and Losick, 2006). In addition, the sensor proteins RetS and LadS regulate T3SS gene expression and biofilm formation in an reciprocal manner

(Ventre et al., 2006). The induction of several genes involved in T3SS by PaeIII could be due to the 1.9 fold increased levels (not shown) of the major regulator gene *exsA* (Yahr and Wolfgang, 2006). As mentioned above, the PaeIII levels are increased in a *rsmA* mutant strain (Fig. 3B, lane 16) but in contrast to this result *rsmA* mutants have been shown to down-regulate T3SS (Brencic and Lory, 2009). This discrepancy could be due to the different growth phases, under which the samples for the transcriptome analysis and the Northern-blot analysis (Fig. 3B) were taken. Moreover, *P. aeruginosa* cells over-express the T3SS when two citrate synthase isoenzymes, GltA and PrpC, are inhibited by mutation, which leads to increased cAMP levels (Rietsch and Mekalanos, 2006). The transcriptome analysis revealed that *gltA* and *prpC* are -1.59 and -3.81 fold down-regulated, respectively. Thus, the results obtained by Rietsch and Melkalanos (2006) can be reconciled with the transcriptome analysis upon *paeIII* over-expression.

The other group of proteins which was induced by PaeIII includes components of the MexX/Y multidrug efflux pump and the regulators MexZ and PA5471 (Fig. 5B; Table 3). MexZ acts as an repressor of *mexX/Y* by binding to a 20 bp palindromic sequence in the 5' UTR of *mexX* (Yamamoto et al., 2009), whereas PA5471 was shown to promote *mexX/Y* expression (Morita et al., 2006). PA5471 interacts with MexZ, blocks its function, and facilitates transcription of the *mexX/Y* operon (Yamamoto *et al.*, 2009). The 4.55-fold increase of PA5471 during over-expression of *paeIII* may therefore alleviate the repressor function of MexZ and may explain the >5- fold increase in MexX/Y (Table 3). The MexX/Y-OprM multidrug efflux pump responds to ribosomes impaired in their function (Jeannot *et al.*, 2005; Caughlan *et al.*, 2009). PA5471 is induced when ribosomes are inactivated by antibiotics, which can cause mistranslation and the production of aberrant polypeptides. Similarily, reactive oxygen species can damage ribosomal components, which again can yield aberrant polypeptides or damage of normal polypeptides (Poole, 2008). A recent study showed that an increased copy number of ORF PA0547 (up-regulated under *paeIII* over-expression; Table 3)

leads to an increased aminoglycoside resistance (Struble and Gill, 2009). Therefore, ORF PA0547 might be involved in the regulation of MexX/Y expression. Possible interaction sites of PaeIII with PA0547 could be discerned using the program TargetRNA (Supplementary Fig. S4A). The possible interaction sites of PaeIII with PA0547 (Supplementary Fig. S4A) is far upstream of the start codon in the 5' UTR. Nonetheless, it was shown recently that binding of a sRNA to an upstream region can regulate the downstream gene (Holmqvist *et al.*, 2010).

The repressed genes encode proteins involved in amino acid biosynthesis and metabolism, and genes that are related to phage (pyocins). In addition, gene functions involved in carbon compound and central intermediary metabolism were as well down-regulated (Fig. 5B, Table 4). Components of R2 and F2 bacteriocins that act against the same or closely related species are termed pyocins, and resemble phage tails (Nakayama *et al.*, 2000). Pyocins are involved in modulating bacterial population dynamics in biofilms, particularly under anaerobic growth conditions, which were shown to induce pyocin production (Waite and Curtis, 2009). Furthermore, pyocins can be induced by hydrogen peroxide. They could serve to kill closely related species or to exert an defense mechanism against oxidative attack by host cells (Chang *et al.*, 2005).

Another potential target is *rpoS* which was 2.9 fold down-regulated upon *paeIII* over-expression (Table 4). In *P. aeruginosa,* RpoS is involved in the regulation of QS *via* RhlR and LasR (Latifi *et al.*, 1996), but it also controls the secretion of extracellular virulence factors such as alginate, exotoxin A, elastases and exoenzyme S (Suh *et al.*, 1999; Sonnleitner *et al.*, 2003; Hogardt *et al.*, 2004). ExoS synthesis is down-regulated in the presence of RpoS, whereas proteins needed for biofilm development are up-regulated (Hogardt *et al.*, 2004). These studies are in agreement with the *paeIII* transcriptome data. Upon over-expression of *paeIII*, T3SS synthesis is increased. In opposite, *rpoS* was decreased, and accordingly genes involved in anaerobic respiration, pyocin production and genes regulated by ANR are down-regulated. The transcription of *rpoS* is controlled by GacA/S, QS and the transcriptional

activator protein PsrA (-1,2 fold) (Bertani *et al.*, 2003; Kojic *et al.*, 2005). When direct PsrA regulated genes (Kojic *et al.*, 2005) are compared to potential PaeIII targets only three overlapping genes (PA2951-53, -1.9, -1.32 and -1.31 fold , respectively) were found. This suggests that PaeIII does not affect *rpoS* expression *via* PsrA.

The synthesis of PA1202 was found to be up-regulated by PaeIII in the proteome analysis (Fig. 4A). However, PA1202 was -2.17 fold repressed in the transcriptome analysis upon *paeIII* over-expression (Table 4). Recently, PA1202 was found to be under control of the bistable expression regulator BexR (-1.52 fold). This regulation apperently results in heterogenous expression of target genes within a cell population by generating diversity in an isogenic population (Turner *et al.*, 2009). This mechanism of regulation could explain the varying effects of PaeIII on PA1202 expression. Another possibility could be that the different means of over-expressing *paeIII*, plasmid-mediated (proteomics) and inducible (transcriptomics), can cause a different outcome with regard to PA1202 synthesis.

Among the down-regulated genes were the *hcnABC*-(Pessi and Haas, 2000) and the *arcABC* operons (Gamper *et al.*, 1991), genes involved in denitrification (*nirNCMS*, n*arK1L*) (Arai *et al.*, 1997; Schreiber *et al.*, 2007) and the cytochrome c peroxidase *ccpR* (Ray and Williams, 1997), all of which are regulated by the anaerobic regulator ANR (Table 3). ANR induces expression of target genes coordinately with the two component regulatory system NarXL (Schreiber *et al.*, 2007), the mRNA levels of which were down-regulated -1.99 fold upon *paeIII* over-expression.

In summary, the transcriptome analysis revealed an induction by PaeIII of genes involved in T3SS, antibiotic resistance, and a repression of genes required for anaerobic respiration as well as for pyocin production. The GacA/S system together with the sensor kinases RetS and LadS plays an important role in the regulation of these gene families (Ventre *et al.*, 2006), . Bioinformatic analysis using TargetRNA (Tjaden *et al.*, 2006) revealed the virulence gene regulator LadS as a potential target of PaeIII, with a possible interaction site overlapping with

the Shine and Dalgarno and the start codon of *ladS* (Fig. 5C). However, the data from transcriptome analysis revealed no changes in *ladS* mRNA levels upon *paeIII* over-expression. However the effects caused by translational regulation on target genes may not always be reflected in the steady state mRNA levels. Base-pairing of a sRNA with a mRNA may block translation without immediate destruction of the mRNA (Caron *et al.*, 2010), which may not be obvious in the transcriptome analysis.

Verification of potential PaeIII targets

Next, we used antibiotic susceptibility tests and transcriptional- and translational-*lacZ* reporter gene fusions to validate potential PaeIII targets predicted by the proteome and the transcriptome analysis. We focussed on regulators of virulence gene expression, such as MexZ, PA5471 and RpoS.

To investigate a possible effect of PaeIII on MexX/Y induced multidrug efflux, the antibiotic susceptibility of PAO1 and PAO1Δ*paeIII* was compared to the *paeIII* over-expressing strain (Fig. 6). MexX/Y is known to respond to antimicrobial agents that target the ribosome (Masuda *et al.*, 2000; Jeannot *et al.*, 2005). The same CFU of strains PAO1 (pME4510), PAO1 (pME-PaeIII) and PAO1Δ*paeIII* (pME4510) were spotted onto LB plates containing two different tetracyclin concentrations. This experiment showed that PAO1Δ*paeIII* (pME4510) and PAO1 (pME4510) were more sensitive to the antbiotic than the *paeIII* over-expressing strain PAO1 (pME-PaeIII) (Fig. 6). Given the results obtained in the transcriptome analysis, we hypothesized that PaeIII induces MexX/Y by controlling one of the regulators of the multidrug efflux pump. Therefore, transcriptional and translational *lacZ*-fusions to *mexZ* and ORF PA5471 were constructed. The transcription and translation of the *mexZ-lacZ* fusion was not changed upon *paeIII* over-expression (data not shown). Expression and drug inducibility of ORF PA5471 requires the entire PA5472-PA5471 intergenic region which also harbours a possible *paeIII* complementarity region (Supplementary Fig. S4B). A 5’ truncation

of 21 nt lead to little or no transcription (Morita *et al.*, 2009). The constructs (pME6014-PA5471 and pME6016-PA5471) used in this study lacked 8 nt of this intergenic region. It contained the entire promoter region as well as the interrupted inverted repeats, which were hypothesized to be necessary for binding of a transcriptional regulator (Morita *et al.*, 2009). However, the β-galactosidase activity conferred by the translational ORF PA5471-*lacZ* fusion in PAO1 as well as in PAO1 over-expressing *paeIII* was very low (data not shown). In addition, no effect of *paeIII* on the transcription of ORF PA5471 using plasmid pME6016-PA5471 could be detected (data not shown). Another possibility is that the aforementioned transcriptional regulator PA0547 (Struble and Gill, 2009), which was 2.13 fold up-regulated upon *paeIII* over-expression (Table 3), is a direct target of *paeIII* and that it can activate MexX/Y expression.

The transcriptome analysis revealed a decrease of the *rpoS* mRNA levels upon *paeIII* over-expression (Table 3). Furthermore, TargetRNA (Tjaden *et al.*, 2006) predicted a possible interaction of the first loop of PaeIII (Fig. 1D) with the Shine and Dalgarno sequence of *rpoS* (Fig. 7C). Transcriptional and translational *lacZ*-fusions to *rpoS* were constructed and the β-galactosidase activity was measured in strains PAO1 and PAO1Δ*paeIII*. In the presence of PaeIII a decraese in translation was observed (Fig 7A), whereas transcription was not affected (Fig. 7B). The comparative analysis of *rpoS* targets (Schuster *et al.*, 2004) and potential *paeIII* targets, revealed by transcriptomics, did not show significant overlaps. Bioinformatic analysis revealed a potential *paeIII* complementarity region in the 5' UTR of *hfq* (Fig. 7D) that covers the Shine and Dalgarno sequence. Hfq has been shown to be required for efficient *rpoS* translation in *E. coli* (Muffler *et al.*, 1996) as well as in *P. aeruginosa* (Sonnleitner *et al.*, 2003). The transcriptome analysis did not reveal any changes in *hfq* mRNA upon *paeIII* over-expression. Nontheless, it is possible that *paeIII* affects RpoS translation *via* Hfq. Also, the fact that the PaeIII steady state levels were decreased in the presence of *hfq* may be explained by an

inhibitory base-pairing interaction between *hfq* mRNA and PaeIII followed by coupled degradation of both.

References

Aiba, H. (2007). "Mechanism of RNA silencing by Hfq-binding small RNAs." Curr Opin Microbiol **10**(2): 134-9.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res **25**(17): 3389-402.

Arai, H., T. Kodama and Y. Igarashi (1997). "Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*." Mol Microbiol **25**(6): 1141-8.

Bertani, I., M. Sevo, M. Kojic and V. Venturi (2003). "Role of GacA, LasI, RhlI, Ppk, PsrA, Vfr and ClpXP in the regulation of the stationary-phase sigma factor *rpoS*/RpoS in *Pseudomonas*." Arch Microbiol **180**(4): 264-71.

Blumer, C., S. Heeb, G. Pessi and D. Haas (1999). "Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites." Proc Natl Acad Sci U S A **96**(24): 14073-8.

Brencic, A. and S. Lory (2009). "Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA." Mol Microbiol **72**(3): 612-32.

Brennan, R. G. and T. M. Link (2007). "Hfq structure, function and ligand binding." Curr Opin Microbiol **10**(2): 125-33.

Burrowes, E., C. Baysse, C. Adams and F. O'Gara (2006). "Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis." Microbiology **152**(Pt 2): 405-18.

Caron, M. P., D. A. Lafontaine and E. Massé (2010). "Small RNA-mediated regulation at the level of transcript stability." RNA Biol **7**(2).

Caughlan, R. E., S. Sriram, D. M. Daigle, A. L. Woods, J. Buco, R. L. Peterson, J. Dzink-Fox, S. Walker and C. R. Dean (2009). "Fmt bypass in *Pseudomonas aeruginosa* causes induction of MexXY efflux pump expression." Antimicrob Agents Chemother **53**(12): 5015-21.

Chang, W., D. A. Small, F. Toghrol and W. E. Bentley (2005). "Microarray analysis of *Pseudomonas aeruginosa* reveals induction of pyocin genes in response to hydrogen peroxide." BMC Genomics **6**: 115.

Colovos, C., D. Cascio and T. O. Yeates (1998). "The 1.8 A crystal structure of the *ycaC* gene product from *Escherichia coli* reveals an octameric hydrolase of unknown specificity." Structure **6**(10): 1329-37.

Costerton, J. W., P. S. Stewart and E. P. Greenberg (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-22.

Dombrecht, B., K. Marchal, J. Vanderleyden and J. Michiels (2002). "Prediction and overview of the RpoN-regulon in closely related species of the Rhizobiales." Genome Biol **3**(12): RESEARCH0076.

Dubnau, D. and R. Losick (2006). "Bistability in bacteria." Mol Microbiol **61**(3): 564-72.

Frangipani, E., V. I. Slaveykova, C. Reimmann and D. Haas (2008). "Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation." J Bacteriol **190**(20): 6706-17.

Gamper, M., A. Zimmermann and D. Haas (1991). "Anaerobic regulation of transcription initiation in the *arcDABC* operon of *Pseudomonas aeruginosa*." J Bacteriol **173**(15): 4742-50.

Gonzalez, N., S. Heeb, C. Valverde, E. Kay, C. Reimmann, T. Junier and D. Haas (2008). "Genome-wide search reveals a novel GacA-regulated small RNA in *Pseudomonas* species." BMC Genomics **9**: 167.

Gruber, A. R., R. Lorenz, S. H. Bernhart, R. Neubock and I. L. Hofacker (2008). "The Vienna RNA websuite." Nucleic Acids Res **36**(Web Server issue): W70-4.

Hauser, A. R. (2009). "The type III secretion system of Pseudomonas aeruginosa: infection by injection." Nat Rev Microbiol **7**(9): 654-65.

Heurlier, K., V. Denervaud, G. Pessi, C. Reimmann and D. Haas (2003). "Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1." J Bacteriol **185**(7): 2227-35.

Hogardt, M., M. Roeder, A. M. Schreff, L. Eberl and J. Heesemann (2004). "Expression of *Pseudomonas aeruginosa exoS* is controlled by quorum sensing and RpoS." Microbiology **150**(Pt 4): 843-51.

Holloway, B. W., V. Krishnapillai and A. F. Morgan (1979). "Chromosomal genetics of *Pseudomonas*." Microbiol Rev **43**(1): 73-102.

Holmqvist, E., J. Reimegard, M. Sterk, N. Grantcharova, U. Romling and E. G. Wagner (2010). "Two antisense RNAs target the transcriptional regulator CsgD to inhibit curli synthesis." Embo J.

Hornef, M. W., A. Roggenkamp, A. M. Geiger, M. Hogardt, C. A. Jacobi and J. Heesemann (2000). "Triggering the ExoS regulon of *Pseudomonas aeruginosa*: A GFP-reporter analysis of exoenzyme (Exo) S, ExoT and ExoU synthesis." Microb Pathog **29**(6): 329-43.

Huerta, A. M. and J. Collado-Vides (2003). "Sigma70 promoters in *Escherichia coli*: specific transcription in dense regions of overlapping promoter-like signals." J Mol Biol **333**(2): 261-78.

Jeannot, K., M. L. Sobel, F. El Garch, K. Poole and P. Plesiat (2005). "Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction." J Bacteriol **187**(15): 5341-6.

Kay, E., B. Humair, V. Denervaud, K. Riedel, S. Spahr, L. Eberl, C. Valverde and D. Haas (2006). "Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*." J Bacteriol **188**(16): 6026-33.

Kojic, M., B. Jovcic, A. Vindigni, F. Odreman and V. Venturi (2005). "Novel target genes of PsrA transcriptional regulator of *Pseudomonas aeruginosa*." FEMS Microbiol Lett **246**(2): 175-81.

Lapouge, K., M. Schubert, F. H. Allain and D. Haas (2008). "Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour." Mol Microbiol **67**(2): 241-53.

Latifi, A., M. Foglino, K. Tanaka, P. Williams and A. Lazdunski (1996). "A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS." Mol Microbiol **21**(6): 1137-46.

Lenz, D. H., K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen and B. L. Bassler (2004). "The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*." Cell **118**(1): 69-82.

Leoni, L., A. Ciervo, N. Orsi and P. Visca (1996). "Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity." J Bacteriol **178**(8): 2299-313.

Lewenza, S., J. L. Gardy, F. S. Brinkman and R. E. Hancock (2005). "Genome-wide identification of Pseudomonas aeruginosa exported proteins using a consensus computational strategy combined with a laboratory-based PhoA fusion screen." Genome Res **15**(2): 321-9.

Lin-Chao, S. and H. Bremer (1986). "Effect of the bacterial growth rate on replication control of plasmid pBR322 in *Escherichia coli*." Mol Gen Genet **203**(1): 143-9.

Livny, J., A. Brencic, S. Lory and M. K. Waldor (2006). "Identification of 17 *Pseudomonas aeruginosa* sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2." Nucleic Acids Res **34**(12): 3484-93.

Martin, D. W., M. J. Schurr, H. Yu and V. Deretic (1994). "Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to sigma E and stress response." J Bacteriol **176**(21): 6688-96.

Massé, E., H. Salvail, G. Desnoyers and M. Arguin (2007). "Small RNAs controlling iron metabolism." Curr Opin Microbiol **10**(2): 140-5.

Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto and T. Nishino (2000). "Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-*oprM* efflux pumps in *Pseudomonas aeruginosa*." Antimicrob Agents Chemother **44**(12): 3322-7.

Miller, J. H. (1972). "Experiments in Molecular Genetics." Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Morita, Y., M. L. Sobel and K. Poole (2006). "Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: involvement of the antibiotic-inducible PA5471 gene product." J Bacteriol **188**(5): 1847-55.

Morita, Y., C. Gilmour, D. Metcalf and K. Poole (2009). "Translational control of the antibiotic inducibility of the PA5471 gene required for *mexXY* multidrug efflux gene expression in *Pseudomonas aeruginosa*." J Bacteriol **191**(15): 4966-75.

Muffler, A., D. Fischer and R. Hengge-Aronis (1996). "The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for *rpoS* translation in *Escherichia coli*." Genes Dev **10**(9): 1143-51.

Nakayama, K., K. Takashima, H. Ishihara, T. Shinomiya, M. Kageyama, S. Kanaya, M. Ohnishi, T. Murata, H. Mori and T. Hayashi (2000). "The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage." Mol Microbiol **38**(2): 213-31.

Nikulin, A., E. Stolboushkina, A. Perederina, I. Vassilieva, U. Bläsi, I. Moll, G. Kachalova, S. Yokoyama, D. Vassylyev, M. Garber and S. Nikonov (2005). "Structure of *Pseudomonas aeruginosa* Hfq protein." Acta Crystallogr D Biol Crystallogr **61**(Pt 2): 141-6.

Oglesby, A. G., J. M. Farrow, 3rd, J. H. Lee, A. P. Tomaras, E. P. Greenberg, E. C. Pesci and M. L. Vasil (2008). "The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing." J Biol Chem **283**(23): 15558-67.

Palmer, K. L., L. M. Aye and M. Whiteley (2007). "Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum." J Bacteriol **189**(22): 8079-87.

Pessi, G. and D. Haas (2000). "Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*." J Bacteriol **182**(24): 6940-9.

Pessi, G. and D. Haas (2001). "Dual control of hydrogen cyanide biosynthesis by the global activator GacA in *Pseudomonas aeruginosa* PAO1." FEMS Microbiol Lett **200**(1): 73-8.

Pessi, G., F. Williams, Z. Hindle, K. Heurlier, M. T. Holden, M. Camara, D. Haas and P. Williams (2001). "The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*." J Bacteriol **183**(22): 6676-83.

Poole, K. (2008). "Bacteria multidrug efflux pumps serve other functions." Microbe **3**(4): 179-185.

Ray, A. and H. D. Williams (1997). "The effects of mutation of the anr gene on the aerobic respiratory chain of *Pseudomonas aeruginosa*." FEMS Microbiol Lett **156**(2): 227-32.

Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino, A. Lazdunski and D. Haas (1997). "The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase." Mol Microbiol **24**(2): 309-19.

Repoila, F., N. Majdalani and S. Gottesman (2003). "Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm." Mol Microbiol **48**(4): 855-61.

Rietsch, A. and J. J. Mekalanos (2006). "Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*." Mol Microbiol **59**(3): 807-20.

Rist, M. and M. A. Kertesz (1998). "Construction of improved plasmid vectors for promoter characterization in Pseudomonas aeruginosa and other gram-negative bacteria." FEMS Microbiol Lett **169**(1): 179-83.

Schnider-Keel, U., A. Seematter, M. Maurhofer, C. Blumer, B. Duffy, C. Gigot-Bonnefoy, C. Reimmann, R. Notz, G. Defago, D. Haas and C. Keel (2000). "Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent Pseudomonas fluorescens CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin." J Bacteriol **182**(5): 1215-25.

Schreiber, K., R. Krieger, B. Benkert, M. Eschbach, H. Arai, M. Schobert and D. Jahn (2007). "The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration." J Bacteriol **189**(11): 4310-4.

Schubert, M., K. Lapouge, O. Duss, F. C. Oberstrass, I. Jelesarov, D. Haas and F. H. Allain (2007). "Molecular basis of messenger RNA recognition by the specific bacterial repressing clamp RsmA/CsrA." Nat Struct Mol Biol **14**(9): 807-13.

Schuster, M., A. C. Hawkins, C. S. Harwood and E. P. Greenberg (2004). "The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing." Mol Microbiol **51**(4): 973-85.

Shevchenko, A., O. N. Jensen, A. V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, H. Boucherie and M. Mann (1996). "Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels." Proc Natl Acad Sci U S A **93**(25): 14440-5.

Simon, R., U. Priefer and A. Puhler (1983). "A Broad Host Range Mobilization System for Invivo Genetic-Engineering - Transposon Mutagenesis in Gram-Negative Bacteria." Bio-Technology **1**(9): 784-791.

Simon, R., M. O'Connell, M. Labes and A. Puhler (1986). "Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria." Methods Enzymol **118**: 640-59.

Sonnleitner, E., S. Hagens, F. Rosenau, S. Wilhelm, A. Habel, K. E. Jäger and U. Bläsi (2003). "Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1." Microb Pathog **35**(5): 217-28.

Sonnleitner, E., M. Schuster, T. Sorger-Domenigg, E. P. Greenberg and U. Bläsi (2006). "Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*." Mol Microbiol **59**(5): 1542-58.

Sonnleitner, E., T. Sorger-Domenigg, M. J. Madej, S. Findeiss, J. Hackermüller, A. Hüttenhofer, P. F. Stadler, U. Bläsi and I. Moll (2008). "Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools." Microbiology **154**(Pt 10): 3175-87.

Sonnleitner, E., L. Abdou and D. Haas (2009). "Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*." Proc Natl Acad Sci U S A.

Sorger-Domenigg, T., E. Sonnleitner, V. R. Kaberdin and U. Bläsi (2007). "Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY." Biochem Biophys Res Commun **352**(3): 769-73.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory and M. V. Olson (2000). "Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen." Nature **406**(6799): 959-64.

Struble, J. M. and R. T. Gill (2009). "Genome-scale identification method applied to find cryptic aminoglycoside resistance genes in *Pseudomonas aeruginosa*." PLoS One **4**(11): e6576.

Suh, S. J., L. Silo-Suh, D. E. Woods, D. J. Hassett, S. E. West and D. E. Ohman (1999). "Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*." J Bacteriol **181**(13): 3890-7.

Tjaden, B., S. S. Goodwin, J. A. Opdyke, M. Guillier, D. X. Fu, S. Gottesman and G. Storz (2006). "Target prediction for small, noncoding RNAs in bacteria." Nucleic Acids Res **34**(9): 2791-802.

Toledo-Arana, A., O. Dussurget, G. Nikitas, N. Sesto, H. Guet-Revillet, D. Balestrino, E. Loh, J. Gripenland, T. Tiensuu, K. Vaitkevicius, M. Barthelemy, M. Vergassola, M. A. Nahori, G. Soubigou, B. Regnault, J. Y. Coppee, M. Lecuit, J. Johansson and P. Cossart (2009). "The *Listeria* transcriptional landscape from saprophytism to virulence." Nature **459**(7249): 950-6.

Turner, K. H., I. Vallet-Gely and S. L. Dove (2009). "Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator." PLoS Genet **5**(12): e1000779.

Valentin-Hansen, P., M. Eriksen and C. Udesen (2004). "The bacterial Sm-like protein Hfq: a key player in RNA transactions." Mol Microbiol **51**(6): 1525-33.

van Delden, C. and B. H. Iglewski (1998). "Cell-to-cell signaling and *Pseudomonas aeruginosa* infections." Emerg Infect Dis **4**(4): 551-60.

Ventre, I., A. L. Goodman, I. Vallet-Gely, P. Vasseur, C. Soscia, S. Molin, S. Bleves, A. Lazdunski, S. Lory and A. Filloux (2006). "Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes." Proc Natl Acad Sci U S A **103**(1): 171-6.

Vogel, J. and K. Papenfort (2006). "Small non-coding RNAs and the bacterial outer membrane." Curr Opin Microbiol **9**(6): 605-11.

Vogel, J. and E. G. Wagner (2007). "Target identification of small noncoding RNAs in bacteria." Curr Opin Microbiol **10**(3): 262-70.

Waite, R. D. and M. A. Curtis (2009). "*Pseudomonas aeruginosa* PAO1 pyocin production affects population dynamics within mixed-culture biofilms." J Bacteriol **191**(4): 1349-54.

Waters, L. S. and G. Storz (2009). "Regulatory RNAs in bacteria." Cell **136**(4): 615-28.

Wilderman, P. J., N. A. Sowa, D. J. FitzGerald, P. C. FitzGerald, S. Gottesman, U. A. Ochsner and M. L. Vasil (2004). "Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis." Proc Natl Acad Sci U S A **101**(26): 9792-7.

Winther-Larsen, H. C., J. M. Blatny, B. Valand, T. Brautaset and S. Valla (2000). "Pm promoter expression mutants and their use in broad-host-range RK2 plasmid vectors." Metab Eng **2**(2): 92-103.

Yahr, T. L. and M. C. Wolfgang (2006). "Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system." Mol Microbiol **62**(3): 631-40.

Yamamoto, M., A. Ueda, M. Kudo, Y. Matsuo, J. Fukushima, T. Nakae, T. Kaneko and Y. Ishigatsubo (2009). "Role of MexZ and PA5471 in transcriptional regulation of *mexXY* in *Pseudomonas aeruginosa*." Microbiology **155**(Pt 10): 3312-21.

Ye, R. W., D. Haas, J. O. Ka, V. Krishnapillai, A. Zimmermann, C. Baird and J. M. Tiedje (1995). "Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr." J Bacteriol **177**(12): 3606-9.

TABLE 1. Bacterial strains and plasmid

Strains/plasmid	Genotype/relevant features	Source/reference
P. aeruginosa		
PAO1	wild-type	(Holloway *et al.*, 1979)
PAO1Δ*paeIII*	in frame deletion of *paeIII*	this study
PAO6261	PAO1Δ*anr*	(Ye *et al.*, 1995)
PAO6281	PAO1 *gacA::aadA*, Sm/Sp^R	(Reimmann *et al.*, 1997)
PAO6358	PAO1Δ*rpoN*	(Heurlier *et al.*, 2003)
PAO6852	PAO1 *algU::*Tc^R	(Martin *et al.*, 1994)
PAO1 *pvdS*-		Haas laboratory
PAO1 *rpoS*$^-$	PAO1 *rpoS101::aacCI*, Gm^R	(Suh *et al.*, 1999)
PAZH13	PAO1Δ*rsmA*	(Pessi *et al.*, 2001)
PAO1*hfq*$^-$	PAO1 *hfq::aadA*, Sm/Sp^R	(Sonnleitner *et al.*, 2003)
E. coli		
Top10	F-, *mcrA*, Δ(*mrr-hsd*RMS-*mcr*BC),p80*lacZ*ΔM15 Δ*lacX*74, *deoR*, *recA1*, *ara*D139Δ(*ara-leu*)7697, *galK*, *rpsL*(StrR), *endA1*, *nupG*	Stratagene
S17-1	*recA1 pro thi*; the *tra* genes of plasmid RP4 are integrated in the chromosome	(Simon *et al.*, 1986)
plasmids		
pGEM-T	A/T cloning vector, Ap^R	Promega
pHFQ$_{Pa}$	T7 expression vector for purification of *P. aeruginosa* Hfq, Ap^R	(Nikulin *et al.*, 2005)
pME4510	Broad-range promoter-probe plasmid, Gm^R	(Rist and Kertesz, 1998)
pME-PaeIII	pME4510 harbouring *paeIII* (authentic promoter)	this study
pJT19	plasmid carrying inducible *Pm/xylS* promoter, Kan^R	(Winther-Larsen *et al.*, 2000)
pJT19-PaeIII	pJT19 harbouring *paeIII*	this study
pSUP202	pBR325, Ap^R, Cm^R, Tc^R, *mob*	(Simon *et al.*, 1983)
pSUP202-PaeIIIup	pSUP202 harbouring *paeIII* upstream region	this study
pSUP202-PaeIIIko	pSUP202-PaeIIIup harbouring *paeIII* downstream region	this study
pME3843	translational *hcnA-lacZ* fusion under *tac* promoter	(Blumer *et al.*, 1999)
pME3846	*rhlI*::*lacZ* translational fusion, Tc^R	(Pessi *et al.*, 2001)
pME6014	cloning vectors for translational *lacZ* fusions, Tc^R	(Schnider-Keel *et al.*, 2000)
pME6016	cloning vectors for transcriptional *lacZ* fusions, Tc^R	(Schnider-Keel *et al.*, 2000)

pME*tac*	pME3846 carrying *tac* promoter in *EcoRI*/ *BamHI*	this study
pME*tac*-PA1202	pME*tac* bearing PA1202 in frame *lacZ*-fusion[a]	this study
pME*tac*-PurE	pME*tac* bearing PA1202 in frame *lacZ*-fusion[a]	this study
pME6014-RpoS	pME6014 bearing RpoS in frame *lacZ*-fusion[a]	this study
pME6016-RpoS	pME6016 bearing RpoS *lacZ*-fusion[b]	this study
pME6014-MexZ	pME6014 bearing MexZ in frame *lacZ*-fusion[a]	this study
pME6016-MexZ	pME6016 bearing MexZ *lacZ*-fusion[b]	this study
pME6014-PA5471	pME6014 bearing PA5471 in frame *lacZ*-fusion[a]	this study
pME6016-PA5471	pME6016 bearing PA5471 *lacZ*-fusion[b]	this study

a. translational gene fusions.
b. transcriptional gene fusions.

TABLE 2. Oligonucleotides used in this study

Name	Sequence[a]	Binding region[b]
I_26	CCCCACACTACCATCGGCGATGCGTCG	6039619-6039645
W_28	CGTGCGGCACGGTGGTGCGGG	3958114-3958094
V_34	GGCACCGGGTCGCGATCAG	3958095- 3958077
W_34	CGCACGAGCCCCGGAGATC	3958109- 3958127
Q_32	AAAAAA**CGATCG**ACGAGCCCCGGAGATCCGCAC	3958112-3958132
R_32	AAAAA**GAATTC**CACACGGGAGAAACACTCGC	3958716-3958697
V_40	TTTTTTTT**ACTAGT**CCAGCGCTGTACTATCCCTTCC	3958051-3958072
J_40	TTTTAAAA**GGTACC**GAAAAGGTGAGCCCCGGAGGT	3958150-3958131
M_31	GC**TCTAGA**TAATACGACTCACTATAGGCCAGCGCTGTACTATCCCTTCCAG	3958051-3958074
N_29	GC**TCTAGA**CCGCTGCAAATATGAGAAAAAGACCTAACT	3957982-3958011
O_29	G**GAATTC**AAAAGGTGAGCCCCGGAGGTGCG	3958150-3958128
B_35	TGTA**GGATCC**TCTTGATGGGCGGCGTGCG	1302589-1302607
C_35	TCTT**CTGCAG**GTCGCTTGTAGGTGAAGGACATG	1302717-1302695
J_39	TTTTACAT**CTGCAG**AACCCATGATCACGCCAACCAGT	6106733-6106755
K_39	TTTTTGTA**GGATCC**CCTCGACGAAGCGTGGCTTGT	6106828-6106808
V_47	TTTT**GAATTC**CTCATTTCCATCGCGAAGCC	616059-6161040
W_47	TTTT**CTGCAG**CGGACAAAGGCTTGATGTA	6160672-6160690
T_47	TTTT**GAATTC**TGTCCCTCGATTCGTGAACTC	2212516-2212536
U_47	TTTT**CTGCAG**CCTCTTTGGTTTTCCTGGCC	2212698-2212679
A_52	AAAA**GGATCC**GTTCCACCCGCAGTTAGTACG	4059497-4059477
B_52	TTTT**CTGCAG**GCGTGTAGTCGATGTGCTTGTG	4058742-4058763
Z_34	TACT**GAATTC**CTTAATGGGCCCGCTAACAGC	
A_35	TTTT**GGATCC**CACACATTATACGAGCCGATG	
B_53	GGCACCGGGTCGCGATCAG	3958095-3958077
C_53	CGCACGAGCCCCGGAGATC	3958109-3958127

a. Restriction sites are highlighted in bold and T7 promoter sequences are underlined.

b. The numbers indicate the binding sites on the chromosome of PAO1 according to the *Pseudomonas* genome database: http://v2.pseudomonas.com.

TABLE 3. Transcripts, which are ≥2-fold up-regulated in PAO1 (pJT-PaeIII)

ID[a]	Fold Change[b]	P-value	Target Description[c]	Gene Name
PA0431	2,13	0,0002	hypothetical protein	---
PA0547	2,13	0,0029	probable transcriptional regulator	---
PA1183	5,11	0,0402	C4-dicarboxylate transport protein	*dctA*
PA1317	3,35	0,0169	cytochrome o ubiquinol oxidase subunit II	*cyoA*
PA1318	2,25	0,0045	cytochrome o ubiquinol oxidase subunit I	*cyoB*
PA1319	2,62	0,0007	cytochrome o ubiquinol oxidase subunit III	*cyoC*
PA1321	2,45	0,0019	cytochrome o ubiquinol oxidase protein CyoE	*cyoE*
PA1696	2,09	0,0003	translocation protein in type III secretion	*pscO*
PA1701	2,27	0,0002	conserved hypothetical protein in type III secretion	---
PA1707	2,12	0,0006	regulatory protein PcrH	*pcrH*
PA1708	2,17	0,0005	translocator protein PopB	*popB*
PA1710	2,90	0,0016	exoenzyme S synthesis protein C precursor	*exsC*
PA1711	4,13	0,0000	hypothetical protein	---
PA1712	2,18	0,0002	exoenzyme S synthesis protein B	*exsB*
PA1714	2,09	0,0002	hypothetical protein	---
PA1718	3,76	0,0002	type III export protein PscE	*pscE*
PA1719	2,02	0,0007	type III export protein PscF	*pscF*
PA1722	2,26	0,0001	type III export protein PscI	*pscI*
PA2018	5,31	0,0000	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter	*mexY*
PA2019	7,25	0,0000	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein precursor	*mexX*
PA2020	2,20	0,0007	probable transcriptional regulator	*mexZ*
PA2459	2,15	0,0013	hypothetical protein	---
PA2840	2,36	0,0050	probable ATP-dependent RNA helicase	---
PA3179	2,64	0,0001	conserved hypothetical protein	---
PA3841	2,46	0,0048	exoenzyme S	*exoS*
PA3842	2,57	0,0003	probable chaperone	---
PA5030	2,43	0,0003	probable major facilitator superfamily (MFS) transporter	---
PA5470	6,76	0,0000	probable peptide chain release factor	---
PA5471	4,55	0,0001	hypothetical protein	---
PA5530	2,64	0,0430	probable MFS dicarboxylate transporter	---
ig	2,24	0,0428	intergenic region 1427453-1428080[d]	---
ig	2,54	0,0002	intergenic region 3874653-3873835[d]	---

a. Gene numbers are taken from the *Pseudomonas* genome project (http://www.pseudomonas.com). ig: intergenic region.
b. The change means increase of the respective transcript levels in strain PAO1 (pJT-PaeIII) when compared to PAO1(pJT19).
c. RND: Resistance-Nodulation-Cell Division, MFS: major facilitator superfamily.
d. Position on the PAO1 genome according to the *Pseudomonas* genome project (http://www.pseudomonas.com).

TABLE 4. Transcripts, which are ≥2-fold down-regulated in PAO1 (pJT-PaeIII)

ID[a]	P-Value	Fold Change[b]	Target Description[c]	Gene Name
PA0141	-2,43	0,1154	conserved hypothetical protein	---
PA0277	-2,05	0,0222	conserved hypothetical protein	---
PA0284	-2,30	0,0026	hypothetical protein	---
PA0298	-2,11	0,0009	probable glutamine synthetase	*spuB*
PA0399	-2,33	0,0083	cystathionine beta-synthase	---
PA0482	-2,42	0,0002	malate synthase G	*glcB*
PA0493	-2,35	0,0003	probable biotin-requiring enzyme	---
PA0495	-2,48	0,0010	hypothetical protein	---
PA0509	-2,02	0,0812	probable c-type cytochrome	*nirN*
PA0515	-2,73	0,1452	probable transcriptional regulator	---
PA0517	-2,34	0,2080	probable c-type cytochrome precursor	*nirC*
PA0518	-2,26	0,1149	cytochrome c-551 precursor	*nirM*
PA0519	-2,34	0,1181	nitrite reductase precursor	*nirS*
PA0537	-2,02	0,0007	conserved hypothetical protein	---
PA0614	-2,34	0,0111	hypothetical protein	---
PA0616	-2,69	0,0001	hypothetical protein	---
PA0618	-2,18	0,0002	probable bacteriophage protein	---
PA0619	-2,04	0,0010	probable bacteriophage protein	---
PA0621	-2,54	0,0011	conserved hypothetical protein	---
PA0622	-2,94	0,0004	probable bacteriophage protein	---
PA0623	-2,45	0,0001	probable bacteriophage protein	---
PA0627	-2,01	0,0002	conserved hypothetical protein	---
PA0633	-3,37	0,0000	hypothetical protein	---
PA0635	-2,23	0,0008	hypothetical protein	---
PA0636	-2,46	0,0002	hypothetical protein	---
PA0638	-2,10	0,0002	probable bacteriophage protein	---
PA0639	-2,48	0,0003	conserved hypothetical protein	---
PA0782	-2,22	0,0083	proline dehydrogenase PutA	*putA*
PA0783	-2,04	0,0007	sodium/proline symporter PutP	*putP*
PA0789	-3,17	0,0007	probable amino acid permease	---
PA0795	-3,81	0,0000	citrate synthase 2	*prpC*
PA0796	-2,35	0,0004	carboxyphosphonoenolpyruvate phosphonomutase	*prpB*
PA0833	-2,20	0,0004	hypothetical protein	---
PA0865	-4,61	0,0003	4-hydroxyphenylpyruvate dioxygenase	*hpd*
PA0870	-2,88	0,0015	aromatic amino acid aminotransferase	*phhC*
PA0871	-2,27	0,0027	pterin-4-alpha-carbinolamine dehydratase	*phhB*
PA0872	-3,07	0,0010	phenylalanine-4-hydroxylase	*phhA*
PA0887	-6,59	0,0001	acetyl-coenzyme A synthetase	*acsA*
PA0888	-2,00	0,0011	arginine/ornithine binding protein AotJ	*aotJ*
PA0895	-2,19	0,0068	N-succinylglutamate 5-semialdehyde dehydrogenase	*aruC*
PA0900	-2,02	0,0109	hypothetical protein	---
PA1070	-2,68	0,0015	branched-chain amino acid transport protein BraG	*braG*
PA1071	-2,22	0,0001	branched-chain amino acid transport protein BraF	*braF*
PA1072	-2,46	0,0015	branched-chain amino acid transport protein BraE	*braE*
PA1073	-3,73	0,0000	branched-chain amino acid transport protein BraD	*braD*

PA1074	-2,27	0,0003	branched-chain amino acid transport protein BraC	*braC*
PA1202	-2,17	0,1335	probable hydrolase	---
PA1288	-2,08	0,0001	probable outer membrane protein precursor	---
PA1337	-3,32	0,0016	glutaminase-asparaginase	*ansB*
PA1339	-2,11	0,0019	probable ATP-binding component of ABC transporter	---
PA1341	-2,25	0,0004	probable permease of ABC transporter	---
PA1342	-2,60	0,0004	probable binding protein component of ABC transporter	---
PA1418	-2,71	0,0006	probable sodium:solute symport protein	---
PA1420	-2,88	0,0001	hypothetical protein	---
PA1421	-5,30	0,0001	guanidinobutyrase	*gbuA*
PA1555	-2,59	0,1115	probable cytochrome c	---
PA1556	-4,27	0,0318	probable cytochrome c oxidase subunit	---
PA1557	-3,72	0,0445	probable cytochrome oxidase subunit (cbb3-type)	---
PA1579	-2,23	0,0001	hypothetical protein	---
PA1818	-3,03	0,0004	probable Orn/Arg/Lys decarboxylase	---
PA1838	-2,31	0,0027	sulfite reductase	*cysI*
PA1984	-7,04	0,0035	probable aldehyde dehydrogenase	---
PA1999	-2,14	0,0743	probable CoA transferase, subunit A	---
PA2008	-3,77	0,0024	fumarylacetoacetase	*fahA*
PA2009	-4,45	0,0066	homogentisate 1,2-dioxygenase	*hmgA*
PA2112	-2,03	0,0309	conserved hypothetical protein	---
PA2193	-3,94	0,0890	hydrogen cyanide synthase HcnA	*hcnA*
PA2194	-2,55	0,1987	hydrogen cyanide synthase HcnB	*hcnB*
PA2195	-2,67	0,2080	hydrogen cyanide synthase HcnC	*hcnC*
PA2204	-3,07	0,0003	probable binding protein component of ABC transporter	---
PA2247	-2,20	0,0574	2-oxoisovalerate dehydrogenase (alpha subunit)	*bkdA1*
PA2248	-2,03	0,0934	2-oxoisovalerate dehydrogenase (beta subunit)	*bkdA2*
PA2381	-2,37	0,1173	hypothetical protein	---
PA2444	-2,10	0,0062	serine hydroxymethyltransferase	*glyA2*
PA2445	-2,51	0,0121	glycine cleavage system protein P2	*gcvP2*
PA2446	-2,53	0,0056	glycine cleavage system protein H2	*gcvH2*
PA2634	-2,14	0,0012	probable isocitrate lyase	*aceA*
PA2639	-2,20	0,0018	NADH dehydrogenase I chain C,D	*nuoD*
PA2649	-2,50	0,0006	NADH dehydrogenase I chain N	*nuoN*
PA2760	-2,11	0,0001	probable outer membrane protein precursor	---
PA3031	-2,19	0,0003	hypothetical protein /// tRNA-Pro	---
PA3038	-4,41	0,0000	probable porin	---
PA3155	-2,06	0,0063	probable aminotransferase WbpE	*wbpE*
PA3187	-3,68	0,0799	probable ATP-binding component of ABC transporter	---
PA3190	-3,76	0,0464	probable binding protein component of ABC sugar transporter	---
PA3195	-2,13	0,0122	glyceraldehyde 3-phosphate dehydrogenase	*gapA*
PA3205	-2,03	0,0034	hypothetical protein	---
PA3227	-2,20	0,0005	peptidyl-prolyl cis-trans isomerase A	*ppiA*
PA3331	-2,39	0,1348	cytochrome P450	---
PA3333	-2,11	0,1526	3-oxoacyl-[acyl-carrier-protein] synthase III	*fabH2*
PA3531	-2,37	0,0047	bacterioferritin	*bfrB*
PA3575	-2,11	0,0125	hypothetical protein	---
PA3622	-2,87	0,0010	sigma factor RpoS	*rpoS*
PA3779	-2,45	0,0001	hypothetical protein	---
PA3837	-2,21	0,0024	probable permease of ABC transporter	---
PA3877	-2,25	0,0369	nitrite extrusion protein 1	*narK1*
PA3915	-2,49	0,0025	molybdopterin biosynthetic protein B1	*moaB1*

PA4015	-2,07	0,0009	conserved hypothetical protein	---
PA4130	-2,03	0,2152	probable sulfite or nitrite reductase	---
PA4131	-5,58	0,0831	probable iron-sulfur protein	---
PA4132	-2,12	0,1703	conserved hypothetical protein	---
PA4133	-3,41	0,1214	cytochrome c oxidase subunit (cbb3-type)	---
PA4211	-2,31	0,1933	probable phenazine biosynthesis protein	*phzB*
PA4236	-2,62	0,0263	catalase	*katA*
PA4310	-2,07	0,0283	chemotactic transducer PctB	*pctB*
PA4430	-2,51	0,0002	probable cytochrome b	---
PA4443	-2,54	0,0001	ATP sulfurylase small subunit	*cysD*
PA4496	-2,76	0,0003	probable binding protein component of ABC transporter	---
PA4500	-3,75	0,0000	probable binding protein component of ABC transporter	---
PA4501	-3,67	0,0000	probable porin	---
PA4502	-5,98	0,0000	probable binding protein component of ABC transporter	---
PA4503	-4,52	0,0000	probable permease of ABC transporter	---
PA4504	-6,58	0,0000	probable permease of ABC transporter	---
PA4505	-4,88	0,0000	probable ATP-binding component of ABC transporter	---
PA4506	-5,75	0,0000	probable ATP-binding component of ABC dipeptide transporter	---
PA4571	-2,57	0,0336	probable cytochrome c	---
PA4587	-2,20	0,1354	cytochrome c551 peroxidase precursor	*ccpR*
PA4675	-2,09	0,0001	probable TonB-dependent receptor	---
PA4714	-2,02	0,0053	conserved hypothetical protein	---
PA4880	-3,43	0,0002	probable bacterioferritin	---
PA5112	-2,34	0,0004	esterase EstA	*estA*
PA5152	-2,23	0,0001	probable ATP-binding component of ABC transporter	---
PA5153	-3,70	0,0000	probable periplasmic binding protein	---
PA5171	-3,25	0,0432	arginine deiminase	*arcA*
PA5172	-4,63	0,0262	ornithine carbamoyltransferase, catabolic	*arcB*
PA5173	-3,58	0,0676	carbamate kinase	*arcC*
PA5212	-2,01	0,0004	hypothetical protein	---
PA5303	-2,53	0,0001	conserved hypothetical protein	---
PA5435	-2,06	0,0031	probable transcarboxylase subunit	---
PA5436	-2,25	0,0137	probable biotin carboxylase subunit of a transcarboxylase	---
PA5445	-2,00	0,0148	probable coenzyme A transferase	---
PA5490	-2,06	0,0023	cytochrome c4 precursor	*cc4*
PA5506	-2,09	0,0015	hypothetical protein	---
PA5507	-2,50	0,0006	hypothetical protein	---
PA5508	-2,08	0,0003	probable glutamine synthetase	---

a. Gene numbers are taken from the *Pseudomonas* genome project (http://www.pseudomonas.com). ig: intergenic region.

b. The change means decrease of the respective transcript levels in strain PAO1 (pJT-PaeIII) when compared to PAO1(pJT19).

c. RND: Resistance-Nodulation-Cell Division, MFS: major facilitator superfamily, ABC: ATP-binding cassette.

Figure Legends

Fig. 1. Genetic organisation, expression and expression signals of *paeIII* and predicted secondary structure of PaeIII. **(A)** PaeIII (black arrow) is located between the genes PA3535 and PA3536 (grey arrows). **(B)** Northern-blot analysis of PAO1 total RNA from cells grown in LB to an OD_{600} of 2 using the strand-specific probe W_28 for PaeIII (Table 2). The sizes of the RNA markers (M) are indicated at the left. The arrow denotes the PaeIII signal. **(C)** Sequence of PaeIII (bold) with predicted promoter region (underlined and in italics), and the transcriptional start site of *paeIII* (underlined, in bold and in italics). The stop codon of PA3535 is underlined. The complementary sequence of primer W_28 is underlined and in bold. The sequence of oligonucleotide N_29 is boxed. 5' and 3' ends mapped by 5'-3' circularization are indicated by arrows. **(D)** Secondary structure prediction of PaeIII using the RNAfold WebServer (Gruber *et al.*, 2008). Base pairing probabilities are indicated by colours according to the spectrum in the left lower corner ranging from 0 (magenta) to 1 (red). .

Fig. 2. Influence of Hfq on PaeIII steady state levels and stability. **(A)** The steady state levels of PaeIII were determined by Northern-blot analysis with total RNA isolated at an OD_{600} of 0.5, 1.5, 2.5 and 3.5. The Northern-blot signals were obtained using radioactively labelled probes W_28 and I_26 against PaeIII and 5S rRNA, respectively (Table 2). **(B)** Relative amounts of PaeIII in PAO1 (◊) and PAO1*hfq*$^-$ (▲). The Northern-blot signals were quantified and normalized to that of 5S rRNA signals. The PaeIII levels in PAO1 at OD_{600}= 0.5 were set to 1. The experiment was performed in duplicate. **(C)** Determination of the stability of PaeIII 0, 10, 30, 45, 60 and 90 min after addition of rifampicin by Northern-blot analysis using probes for PaeIII and 5S rRNA.

Fig. 3. Influence of growth and stress on PaeIII levels. **(A)** Determination of the steady state levels of PaeIII by Northern-blot analysis under different growth and stress conditions (lane 1: LB OD_{600}=1, lane 2: BM2 minimal medium OD_{600}=1, lane 3: LB OD_{600}=3, lane 4: oxidative stress, lane 5: osmotic stress, lane 6: heat stress, lane 7: cold stress, lane 8: iron depletion and lane 9: low pH). For the detection of PaeIII the probe W_28 was used and as an internal control probe I_26 for 5S rRNA. **(B)** Steady state levels of PaeIII in different PAO1 mutant strains at OD_{600}=1 (black bars) and at OD_{600}=3 (white bars) grown in LB medium (lanes 1,2,5-18) or synthetic sputum medium (lanes 3 and 4). **(C)** Steady-state levels of PaeIII in the presence (lanes 1 and 2; black bar and vertical stripes) or absence of Hfq (lanes 3 and 4; white bar and horizontal stripes) at an OD_{600} of 2 upon growth in LB medium (lanes 1 and 3) and after serum exposure (lanes 2 and 4) (Sonnleitner *et al.*, 2008, see Materials and Methods), respectively.

Fig. 4. Identification of putative PaeIII targets by proteomics. The protein pattern of PAO1 (pME-PaeIII) (left panel) was compared to that of PAO1 (pME4510) (right panel). The samples were taken at an OD_{600} of 2 upon growth in LB. **(A)** The selected spot (circles), which was increased when PaeIII was over-expressed, was excised and identified by mass-spectrometry as PA1202. **(B)** The selected spots, which were decreased (dotted circles) when PaeIII was over-expressed were identified by mass-spectrometry as PurE (dotted circle 1) and PA4739 (dotted circle 2). Only the relevant sections of the gels are shown.

Fig. 5. Microarray analysis reveals putative PaeIII targets. **(A)** Over-expression of *paeIII* detected by Northern-blot analysis. in strains PAO1 (pJT19) and PAO1 (pJT-PaeIII) 0, 15 and 30 min after addition of 2 mM toluic acid. The Northern-blot signals were obtained using probes against PaeIII and 5S rRNA as described in the legend to Fig. 2. **(B)** The functional classes (www.pseudomonas.com) of genes that are activated (black bars) or repressed (white

bars) after *paeIII* over-expression are shown. Only genes with a minimum fold-change of ≥2 are included. **(C)** Possible interaction site of *ladS* and PaeIII revealed by TargetRNA (Tjaden *et al.*, 2006). Numbers mark the distance from the transcriptional start for *paeIII* (+1) and the distance from the A (+1) of the start codon of *ladS*. The start codon is underlined.

Fig. 6. PaeIII increases antibiotic susceptibility. Growth of PAO1 (pME4510), PAO1 (pME-PaeIII) and PAO1Δ*paeIII* (pME4510) on LB agar plates containing 8 mg/l or 16 mg/l tetracyclin. Dilutions with the same optical density of exponentially growing cultures were spotted and incubated overnight at 37°C.

Fig. 7. PaeIII decreases RpoS translation. **(A)** β- galactosidase activity of a translational *rpoS::lacZ*-fusion (pME6014-RpoS) measured throughout growth in the PAO1 (■) and PAO1Δ*paeIII* (Δ) grown in LB medium. **(B)** β- galactosidase activity of a transcriptional *rpoS-lacZ*-fusion (pME6016-RpoS) measured throughout growth in PAO1 (■) and PAO1Δ*paeIII* (Δ) grown in LB medium. **(C)** Complementarity identified by TargetRNA software (Tjaden *et al.*, 2006) between the *rpoS* 5' UTR and PaeIII. Numbers mark the distance from the transcriptional start of *paeIII* (+1) and the distance from the A (+1) of the start codon *rpoS*. The start codon is underlined. **(D)** Complementarity identified by TargetRNA software (Tjaden *et al.*, 2006) between *hfq* 5' UTR and *paeIII*. Numbers mark the distance from the transcriptional start of *paeIII* (+1) and the distance from the A (+1) of the start codon of *hfq*.

Supplementary Fig. S1. PaeIII binds to Hfq. Labelled RNA (5 nM) was incubated in the absence (lane 1) or in the presence of 10 nM (lane 2), 25 nM (lane 3), 50 nM (lane 4), 100 nM (lane 5), 250 nM (lane 6), 350nM (lane 7) or 500 nM (lane 8) Hfq_6. The Hfq_6:RNA ratio is indicated at the top. Unlabelled tRNA (lane 9 and 10) and PaeIII (lane 11 and 12) were added as competitors. The position of free (F) and Hfq-bound (B) RNA is indicated.

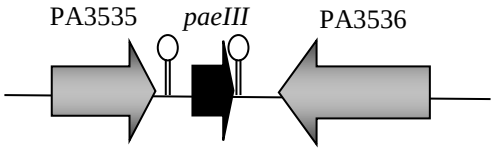
Supplementary Fig. S2. Steady state levels of PaeIII determined by Northern-blot analysis from total RNA isolated at an OD_{600} of 0.7, 2 and 4 from the indicated PAO1 strains. PaeIII and 5S rRNA were detected with probes against PaeIII and 5S rRNA as specified in the legend to Fig. 2.

Supplementary Fig. S3. (A) β- galactosidase activity of a translational PA1202 *lacZ*-fusion measured during growth in PAO1 (pME4510) (□) and upon over-expression of *paeIII* in strain PAO1 (pME-PaeIII) (♦) grown in LB medium. **(B)** β- galactosidase activity of a translational *purE lacZ*-fusion during growth in LB medium in the PAO1 (pME4510) (□) and upon over-expression of *paeIII* in strain PAO1 (pME-PaeIII) (♦).

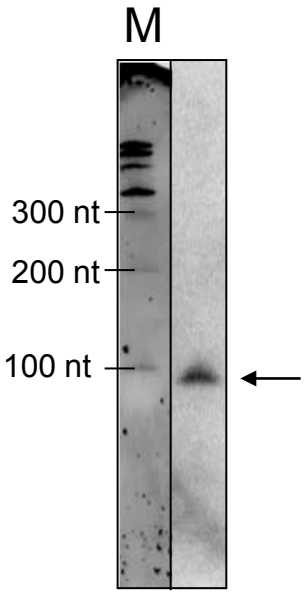
Supplementary Fig. S4. Complementarity identified by TargetRNA software (Tjaden *et al.*, 2006) between the 5' UTR of PA0547 **(A)** and PA5471 **(B)** respectively, with PaeIII. Numbers mark the distance from the transcriptional start of *paeIII* (+1) and the distance from the A (+1) of the start codon of PA0547 and PA5471.

Figure 1

A



B



C

GTCGACTGGCGTTTCTGACTTCGGTTACGAGGGCATGCCAGCCGTTGG

ATGGGTTCCGGCGGGCGGCAAGGGGAAAAAGCCACTTCATTGCCCTCG

-35

GTCACGAAACCGCTGCAAATATGAGAAAAAGACCTAACTAAAGGA*TTCA*

-10

*TG*CGAAATGCATAGCGC*TATACT*GTCCATCC***A*GCGCTGTACTATCCCTT**

CCAGCGCTGATCGCGACCCGGTGCCCGCACCACCGTGCCGCACGAGC

CCCGGAGATCCGCACCTCCGGGGCTCACCTTTT

D

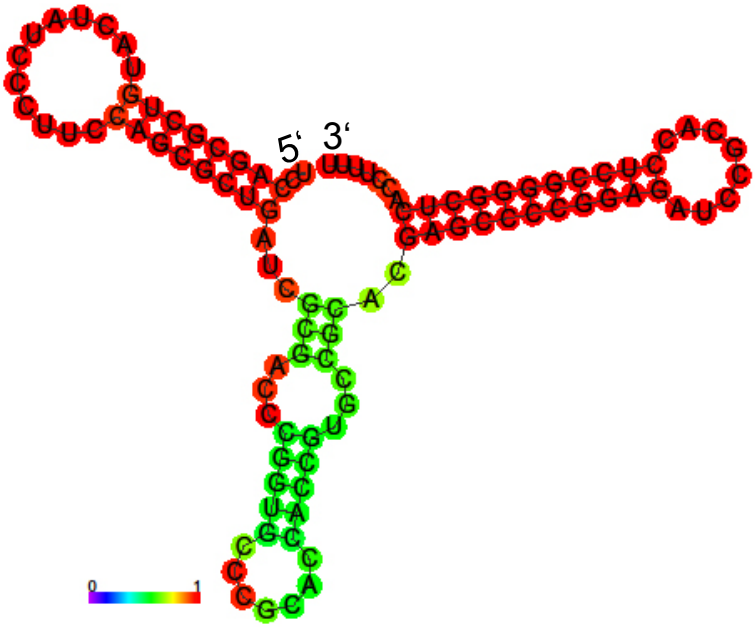
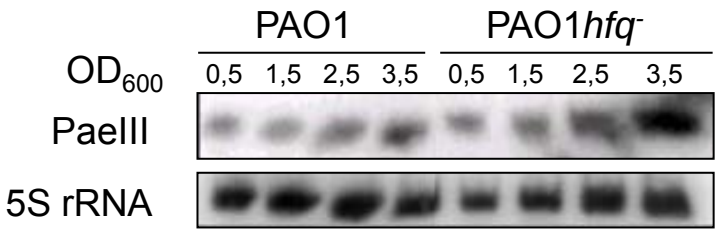
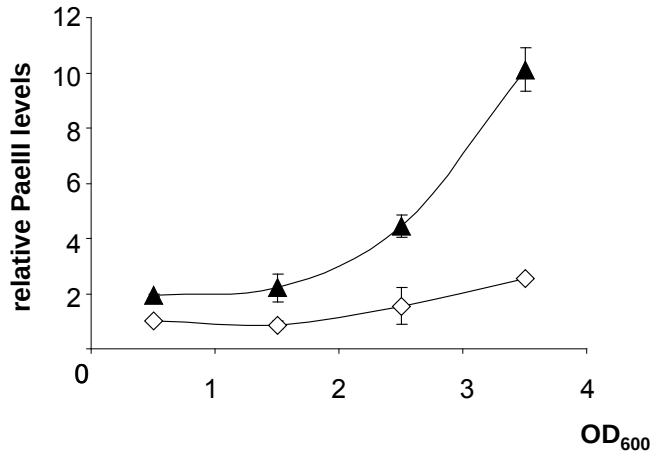


Figure 2

A


PAO1
PAO1*hfq*-
OD600
0,5 1,5 2,5 3,5 0,5 1,5 2,5 3,5
PaeIII
5S rRNA

B


relative PaeIII levels
12
10
8
6
4
2
0
1
2
3
4
OD600

C

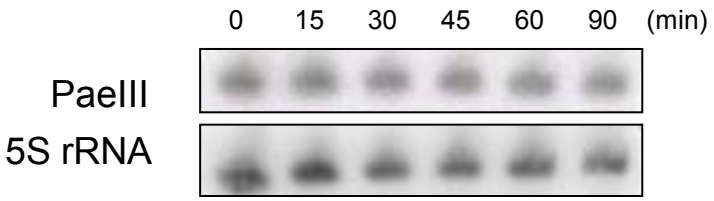
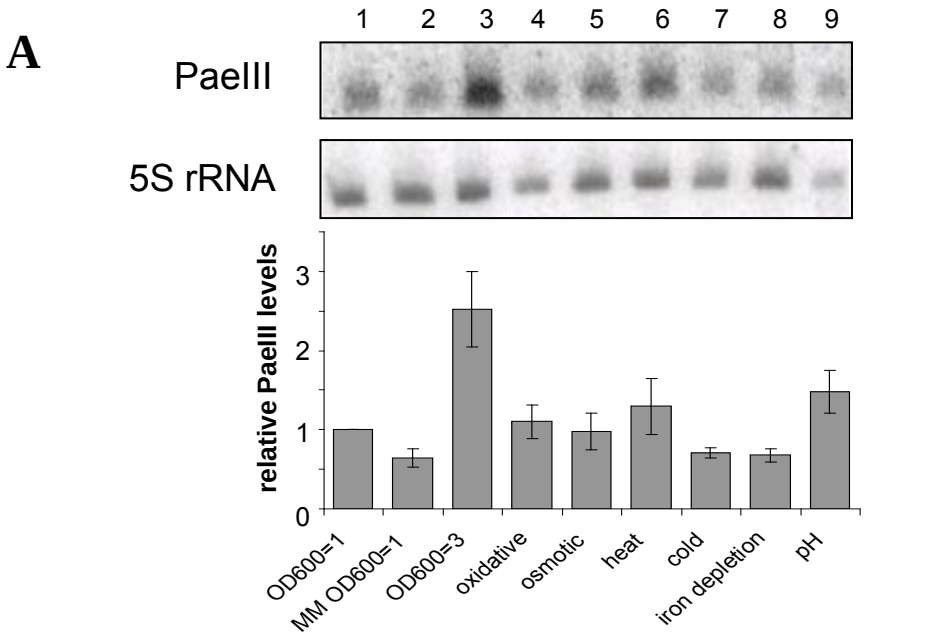
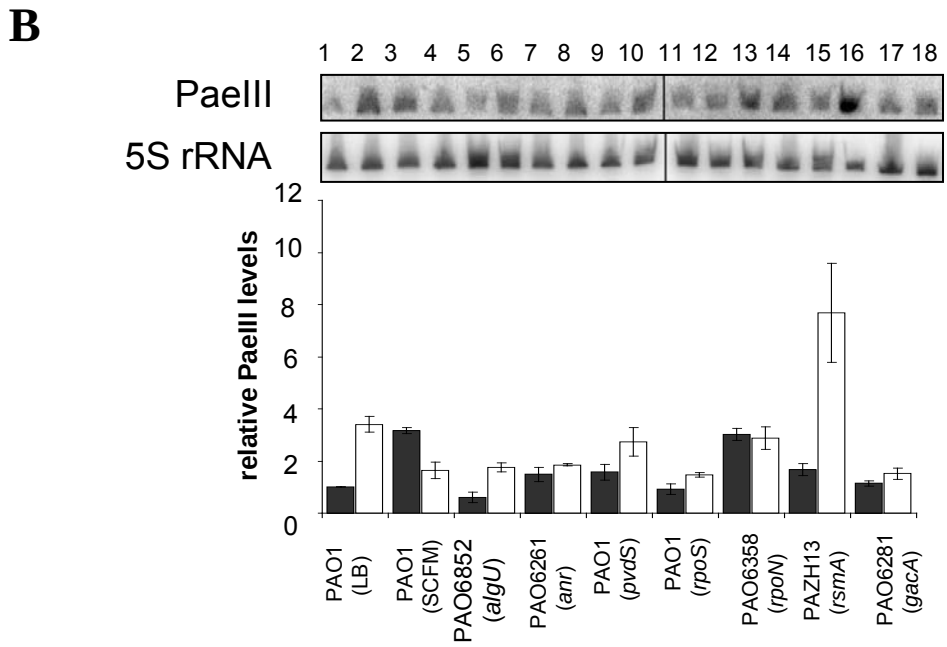

0 15 30 45 60 90 (min)
PaeIII
5S rRNA

Figure 3


A
1 2 3 4 5 6 7 8 9
PaeIII
5S rRNA
relative PaeIII levels
0 1 2 3
OD600=1
MM OD600=1
OD600=3
oxidative
osmotic
heat
cold
iron depletion
pH


B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
PaeIII
5S rRNA
relative PaeIII levels
0 2 4 6 8 10 12
PAO1 (LB)
PAO1 (SCFM)
PAO6852 (algU)
PAO6261 (anr)
PAO1 (pvdS)
PAO1 (rpoS)
PAO6358 (rpoN)
PAZH13 (rsmA)
PAO6281 (gacA)

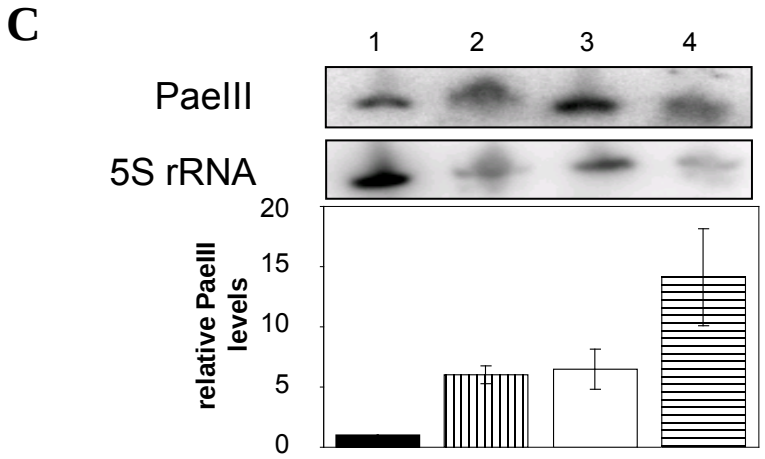

C
1 2 3 4
PaeIII
5S rRNA
relative PaeIII levels
0 5 10 15 20

Figure 4

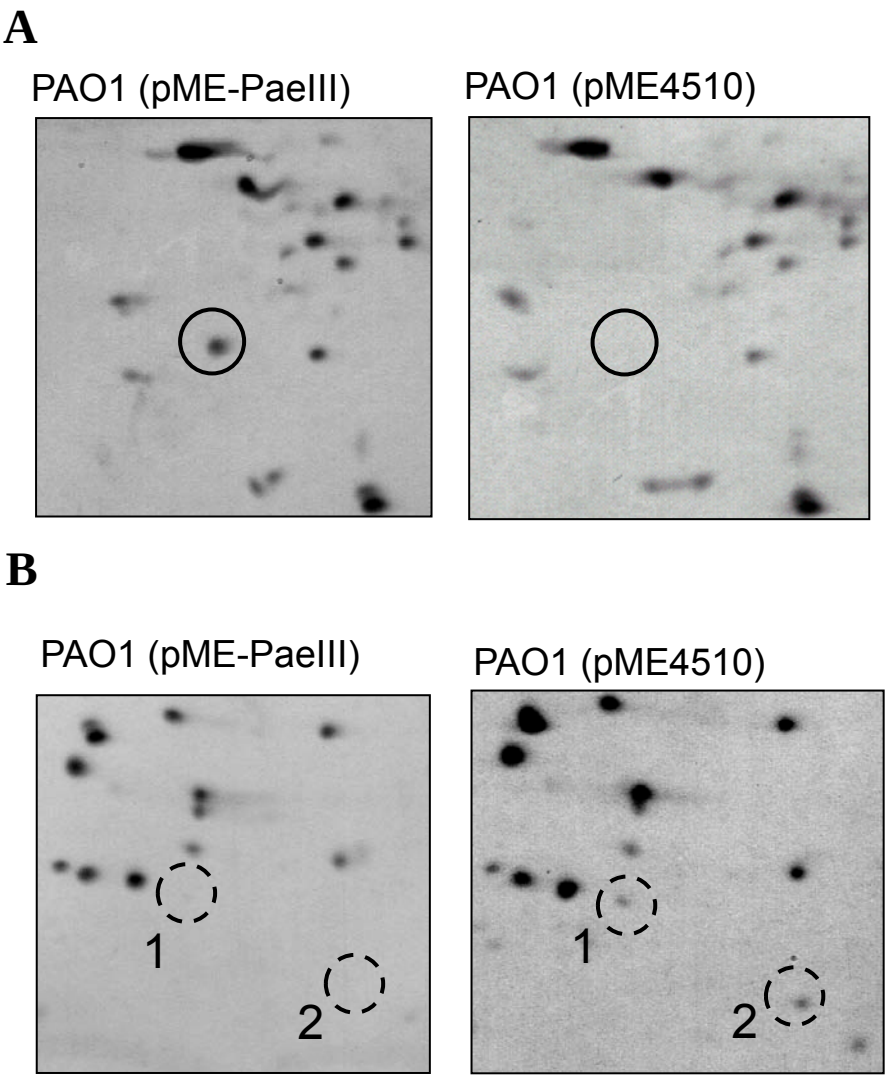
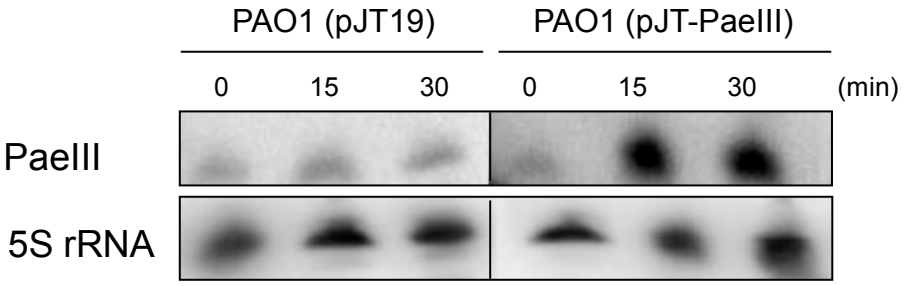
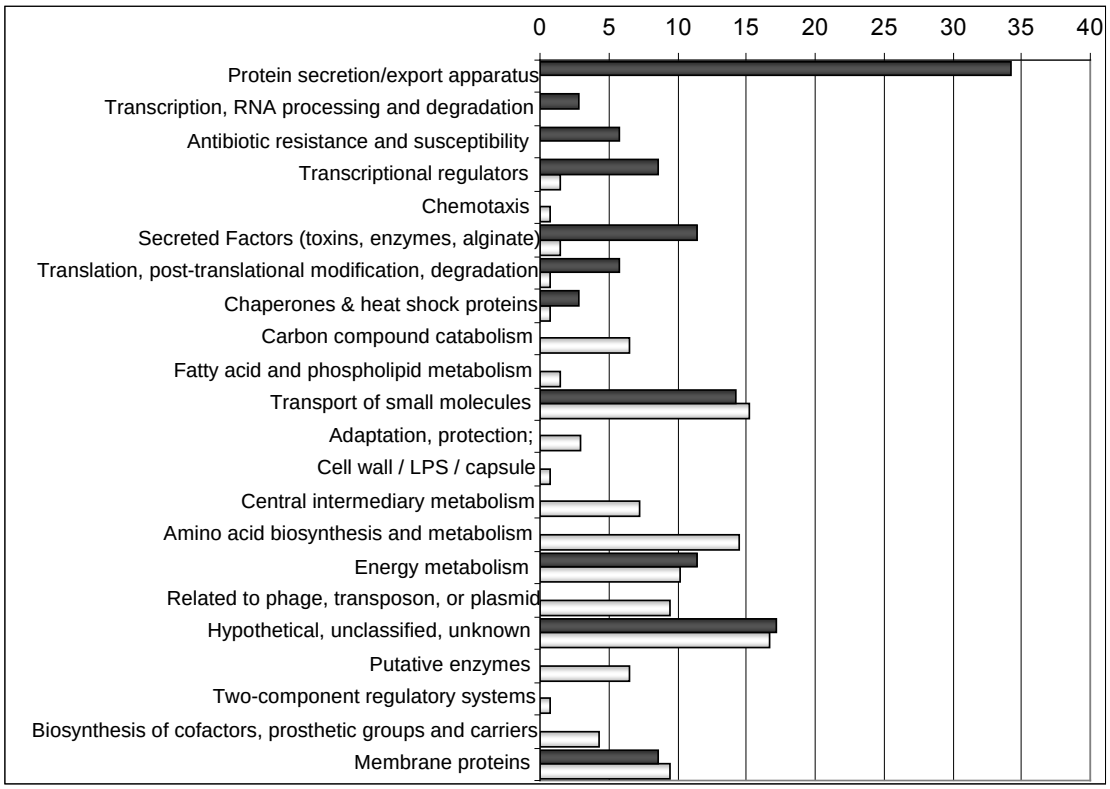

A
PAO1 (pME-PaeIII)
PAO1 (pME4510)
B
PAO1 (pME-PaeIII)
PAO1 (pME4510)
1
2
1
2

Figure 5

A


PAO1 (pJT19)
PAO1 (pJT-PaeIII)
0
15
30
0
15
30
(min)
PaeIII
5S rRNA

B


0
5
10
15
20
25
30
35
40
Protein secretion/export apparatus
Transcription, RNA processing and degradation
Antibiotic resistance and susceptibility
Transcriptional regulators
Chemotaxis
Secreted Factors (toxins, enzymes, alginate)
Translation, post-translational modification, degradation
Chaperones & heat shock proteins
Carbon compound catabolism
Fatty acid and phospholipid metabolism
Transport of small molecules
Adaptation, protection;
Cell wall / LPS / capsule
Central intermediary metabolism
Amino acid biosynthesis and metabolism
Energy metabolism
Related to phage, transposon, or plasmid
Hypothetical, unclassified, unknown
Putative enzymes
Two-component regulatory systems
Biosynthesis of cofactors, prosthetic groups and carriers
Membrane proteins

C

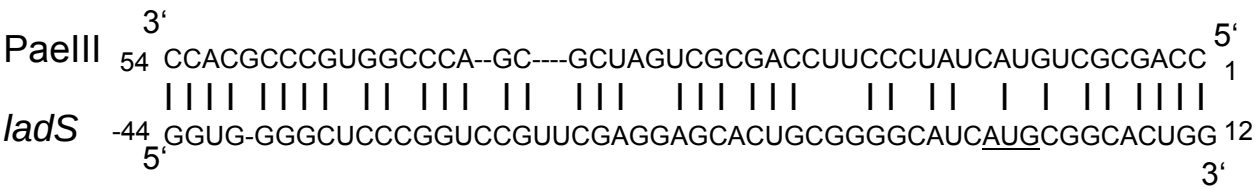

PaeIII
3‘
54 CCACGCCCGUGGCCCA--GC----GCUAGUCGCGACCUUCCCUAUCAUGUCGCGACC 1
5‘
ladS
-44 GGUG-GGGCUCCCGGUCCGUUCGAGGAGCACUGCGGGGCAUCAUGCGGCACUGG 12
5‘
3‘

Figure 6

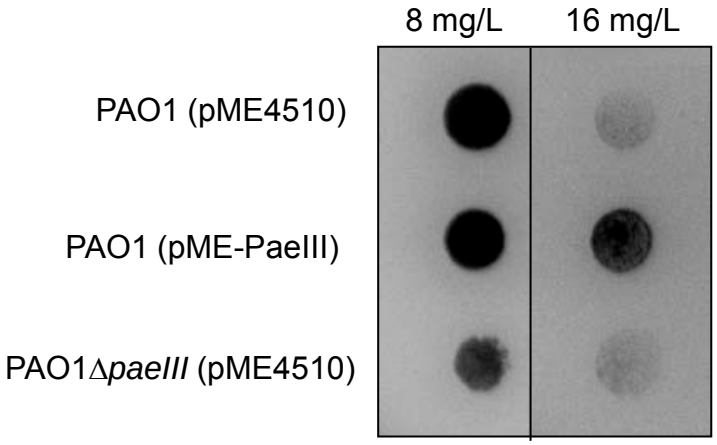
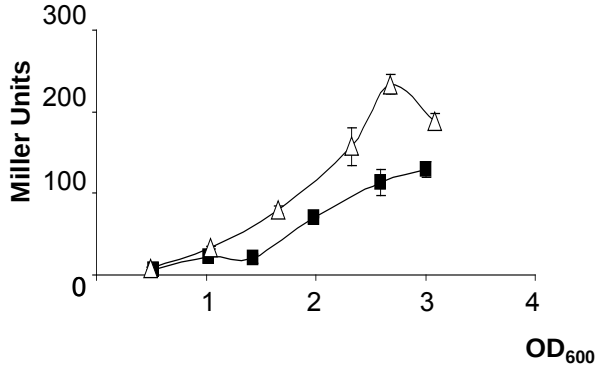
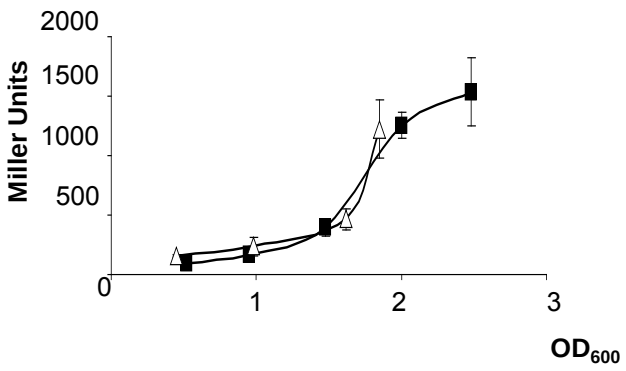

8 mg/L
16 mg/L
PAO1 (pME4510)
PAO1 (pME-PaeIII)
PAO1Δ*paeIII* (pME4510)

Figure 7

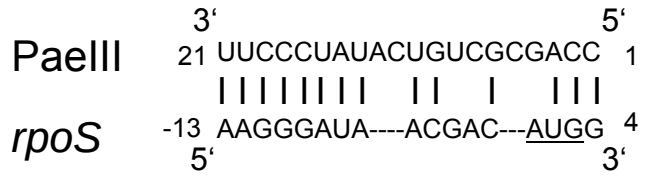
A



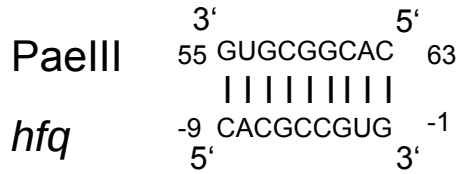
B



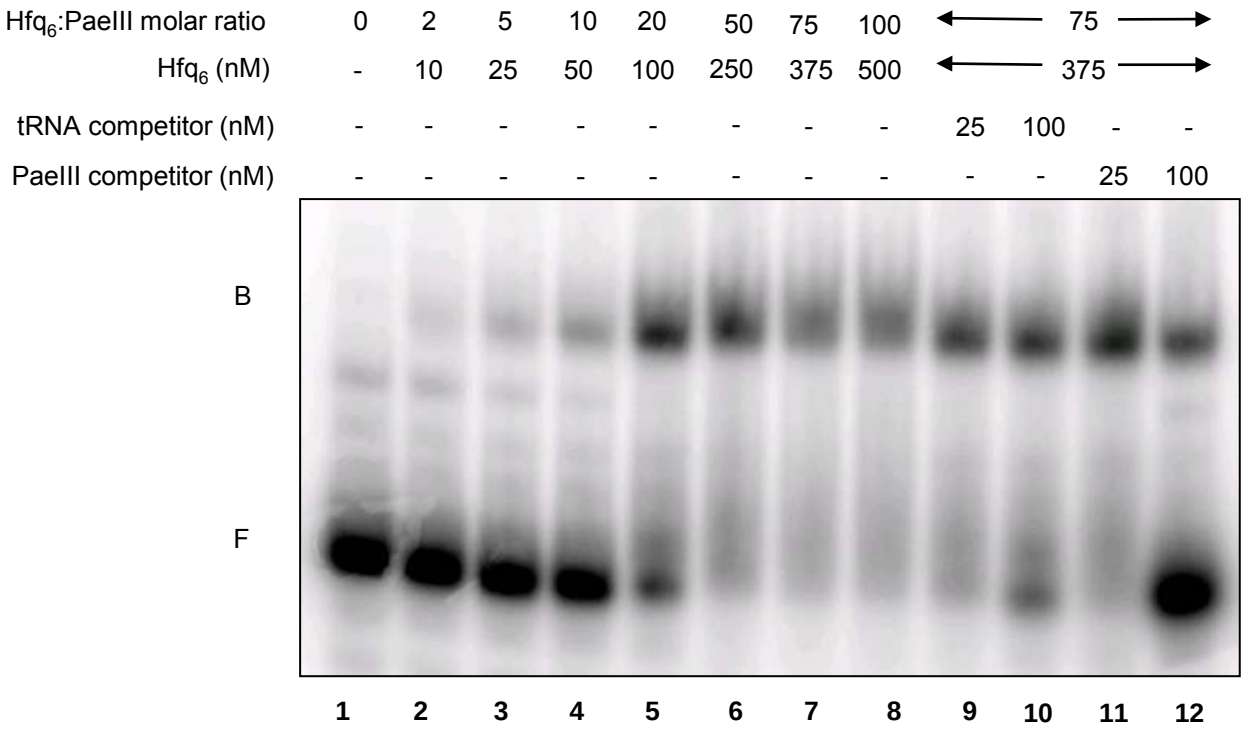
C



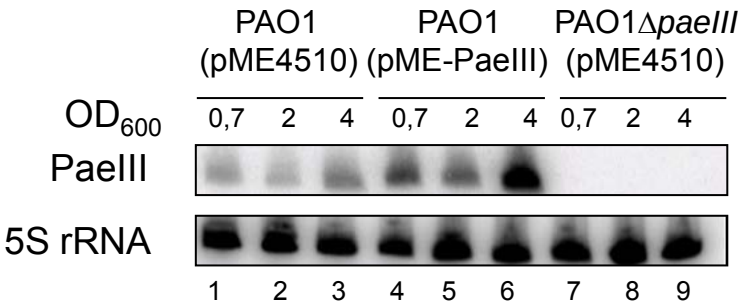
D



Supplementary Figure S1

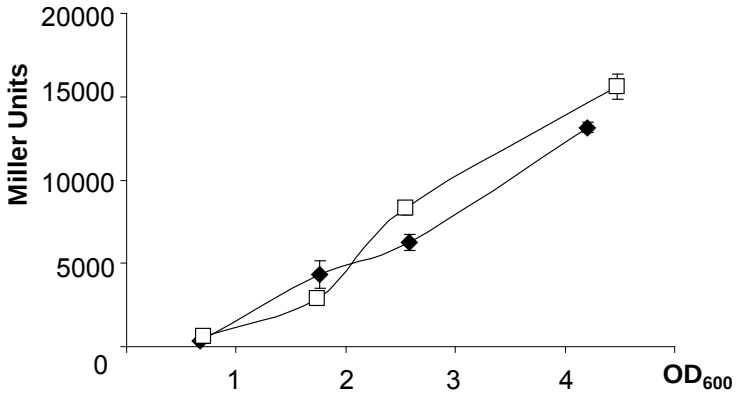


Supplementary Figure S2

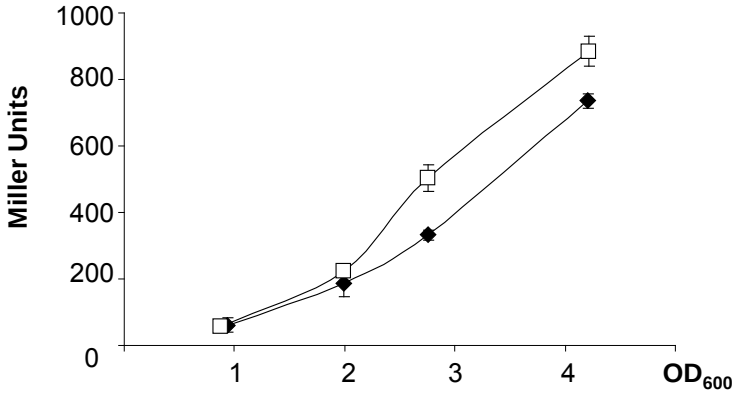


Supplementary Figure S3

A

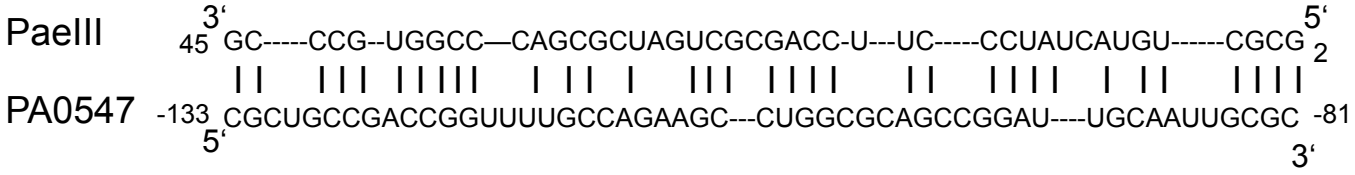


B

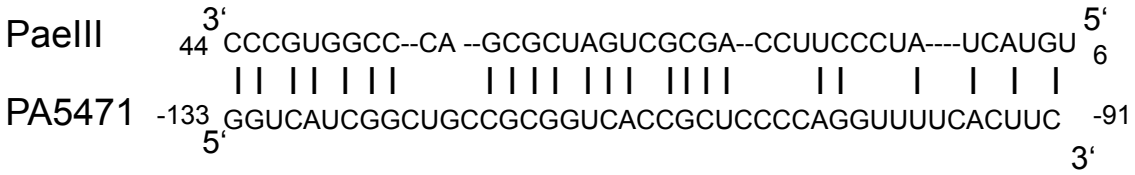


Supplementary Figure S4

A



B



4.4 Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 352 (2007) 769–773
BBRC
www.elsevier.com/locate/ybbrc

Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY

Theresa Sorger-Domenigg, Elisabeth Sonnleitner, Vladimir R. Kaberdin, Udo Bläsi *

Max F. Perutz Laboratories, Department of Microbiology and Immunobiology, University of Vienna, Campus Vienna Biocenter, Dr. Bohrgasse 9, 1030 Vienna, Austria

Received 7 November 2006
Available online 27 November 2006

Abstract

In *Pseudomonas aeruginosa* the Rsm system is involved in regulation of quorum-sensing and virulence gene expression. Our recent studies revealed that the stability and abundance of the non-coding RNA RsmY, which antagonizes the translational regulator RsmA, is dependent on Hfq. Here, we show that Hfq and RsmA bind concurrently to RsmY. Enzymatic probing of RsmY RNA in the presence of RsmA and Hfq verified the proposed –GGA– motifs as RsmA binding sites and located Hfq binding sites in single-stranded regions adjacent to stem-loop structures, respectively. We conclude that distinct binding of Hfq and RsmA on RsmY RNA permits RsmY-mediated RsmA titration upon binding to and stabilization of RsmY RNA by Hfq. In addition, we provide evidence that Hfq sequesters RNase E cleavage sites on RsmY, which explains the previously observed dependence of RsmY RNA stability on Hfq.

Keywords: Hfq; Non-coding RNA; *P. aeruginosa*; RsmA; RsmY

The pathogenicity of *Pseudomonas aeruginosa* can be ascribed to number of intra- and extra-cellular virulence factors, the regulation of which is governed by several interlinked systems, including quorum-sensing (QS), the GacS–GacA two-component system, alternative sigma factors (RpoS, RpoN) as well as the translational repressor protein RsmA [1]. In *P. aeruginosa* the phosphorylated response regulator GacA activates transcription of the small non-coding RNA (ncRNA) genes *rsmY* and *rsmZ* [2,3], which are predominantly expressed during stationary phase [2–4]. These ncRNAs bind to the translational repressor protein RsmA and antagonize its negative effect on synthesis of virulence factors [2,3,5], including protease, elastase, staphylolytic activity (LasA protease), cytotoxic PA-IL lectin, hydrogen cyanide, and the phenanzine pigment pyocyanin [5]. Like its *Escherichia coli* homologue CsrA [6], the *Pseudomonas fluorescens* RsmA homo-dimer binds to GGA-motifs in RsmY and RsmZ RNA, which are located in the loops of secondary structures [7], and it was shown that binding of RsmA/E elevates the stability of RsmY/Z [8]. In *P. aeruginosa* RsmY RNA these GGA-motifs are present as well and it has been suggested that they are required for RsmA binding [9].

Hfq is a global regulatory protein [10], which forms homo-hexameric rings [11–13]. Major physiological tasks of Hfq include stability control of small regulatory RNAs [14,15] and mRNAs [16] as well as positive or negative translational regulation of target mRNAs by small non-coding RNAs [17,18]. In some cases Hfq binding sites have been shown to coincide with RNase E cleavage sites, which can be reconciled with the stabilizing effect of Hfq on small regulatory RNAs [15,19].

A *P. aeruginosa hfq*$^-$ mutant strain showed attenuated virulence, which could be partially ascribed to positive regulation by Hfq of the *rpoS* gene, encoding the stationary phase sigma factor [20] and by Hfq-mediated regulation of QS-controlled genes by RsmY-mediated indirect positive regulation of the QS regulator RhlI [21]. In addition, these studies revealed that Hfq stabilizes RsmY RNA [21].

* Corresponding author. Fax: +43 1 4277 9546.
E-mail address: Udo.Blaesi@univie.ac.at (U. Bläsi).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.084

Here, we show that Hfq and RsmA bind concomitantly to RsmY RNA at distinct as well as overlapping sites and that Hfq can potentially inhibit RNase E cleavage of RsmY RNA. The distinct binding sites are in agreement with a recently proposed model [21] for Hfq-mediated positive control of the autoinducer C4-HSL synthetase, RhlI.

Materials and methods

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. Unless indicated otherwise, bacterial cultures were grown at 37 °C in Luria–Bertani (LB) medium [22] supplemented with appropriate antibiotics.

Gel-mobility shift assays. *Pseudomonas aeruginosa* Hfq and RsmA proteins were purified using *E. coli* strain BL21(DE3) upon plasmid-mediated (Table 1) over-expression of the corresponding genes as recently described [21]. Gel-purified RsmY RNA was 5′-end labelled with [γ-^{32}P]-ATP (Amersham) and re-purified on a 6% polyacrylamide-8 M urea gel [21]. Labelled RNA (0.05 pmol) was first incubated at room temperature for 5 min to refold, then for 15 min with purified *P. aeruginosa* Hfq-hexamer [21] (Hfq_6) (0.5 or 1 pmol) or purified RsmA [21] (2.5 pmol) proteins in 10 μl VD buffer (10 mM Tris, pH 7.4, 60 mM NH_4Cl, 6 mM β-mercaptoethanol, and 2 M Mg-acetate). Concominant binding of Hfq and RsmA was tested by addition of either Hfq_6 (0.25, 0.5, 0.75, 1 or 2 pmol) or RsmA (0.25, 0.5, 1, 2 or 4 pmol) to pre-formed RsmA–RsmY or Hfq–RsmY complexes for 15 min at 4 °C as specified in Fig. 1. Immediately before loading, the samples were mixed with 4 μl of loading dye (75% glycerol, 0.01% xylencyanol, 0.01% bromphenol blue), and loaded on a native 4% polyacrylamide gel. Electrophoresis was performed in TAE buffer at 160 V at 4 °C. The radioactively labelled bands were visualized with a PhosphoImager (Molecular Dynamics).

Enzymatic probing. Aliquots containing 0.16 pmol of 5′-[^{32}P]-labelled RsmY RNA [21] were either added to Hfq_6 (0.8, 2.4 or 4.8 pmol) or RsmA (3.2, 6.4 or 12.8 pmol) and incubated for 20 min at room temperature in VD buffer. Then 1 U endoribonuclease T_1 (Boehringer) or 1 U endoribonuclease T_2 (Sigma–Aldrich) was added and incubation at room temperature was continued for 10 and 4 min, respectively. T_1 and T_2 cleavage reactions were stopped by addition of one volume phenol/chloroform. After phenol/chloroform extraction, the RNA was precipitated with two volumes of ethanol, and loaded on 10% polyacryamide gels containing 8 M urea. A sequence ladder was obtained by T_1 digestion under denaturing conditions as described [23]. The radioactively labelled bands were visualized with a PhosphoImager (Molecular Dynamics).

In vitro RNase E cleavage. 0.16 pmol of 5′-[^{32}P]-labelled RsmY RNA, refolded 5 min at room temperature, was incubated with Hfq_6 (0.8, 2.4 or 4.8 pmol) for 15 min at room temperature. The RNA was first incubated with 25 ng of purified N-terminal fragment of *E. coli* RNaseE (Rne498) [24] in VD buffer in the presence of 1 mM EGTA. The cleavage reaction was started by addition of MgOAc to a final concentration of 5 mM and was continued for 30 min at 37 °C. The cleavage reaction was quenched by addition of 1 μl of 1 μg/μl glycogen in 250 mM EDTA solution. After phenol extraction, the RNA was precipitated, and analyzed on a 10% polyacrylamide-8 M urea gel. The radioactively labelled bands were visualized with a PhosphoImager (Molecular Dynamics).

Table 1
Bacterial strains and plasmid

Strains/ plasmid	Genotype/relevant features	Source/ reference
Pseudomonas aeruginosa		
PAO1	Wild-type	[30]
Escherichia coli		
BL21(DE3)	*E. coli* B, F^-, *dcm*, *ompT*, *hsdS*($r_B^-m_B^-$) *gal*λ(DE3),	Stratagene
Plasmids		
$pHFQ_{Pa}$	T7 expression vector for purification of *P. aeruginosa* Hfq, Ap^R	[12]
pET-*rsmA*	pET22b(+) encodes RsmA with a C-terminal His_6-tag, Ap^R	[21]

Results and discussion

Concomitant binding of Hfq and RsmA to RsmY RNA

A model, devised to explain the Hfq-mediated positive effects on the stability of RsmY and on *rhlI* expression, suggested that Hfq and RsmA bind concomitantly to RsmY RNA [21]. To obtain further support for this model, 5′-[^{32}P]-labelled RsmY RNA was pre-incubated with Hfq (Fig. 1, lanes 4–8) or RsmA (Fig. 1, lanes 10–14), and then increasing concentrations of RsmA or Hfq were added. In

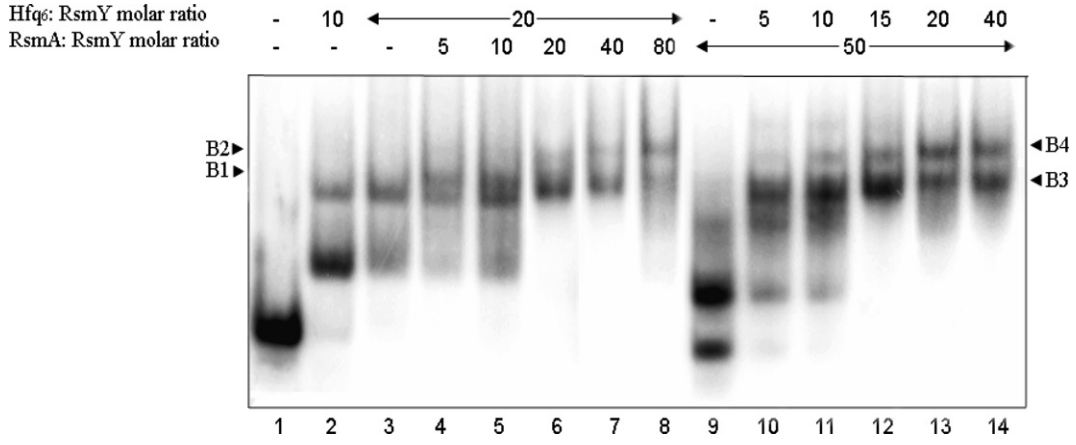


Fig. 1. Concomitant binding of RsmA and Hfq to RsmY RNA. 5′-End-labelled RNA (0.05 pmol) RNA was incubated with either Hfq or RsmA at the molar ratios as indicated on top of the autoradiograph. Then, increasing amounts of either RsmA (lanes 4–8) or Hfq_6 (lanes 10–14) were added to the pre-formed Hfq–RsmY or RsmA–RsmY ribonucleoprotein complexes, respectively, at the molar ratios (protein:RNA) specified on top of the autoradiograph. Lanes 1–3 and 9, controls. The positions of RsmA–RsmY–Hfq complex and of Hfq_6–RsmY–RsmA complex are indicated with B1/B2 and B3/B4, respectively.

either case, the addition of RsmA or Hfq resulted in additional retardation of the ribonucleoprotein complexes (Fig. 1). Two additional bands appeared upon addition of increasing amounts of RsmA and Hfq to pre-formed RsmY–Hfq complexes (Fig. 1; B1 and B2) and to RsmY–RsmA complexes (Fig. 1; B3 and B4). These results supported the idea that RsmA and Hfq bind concomitantly to RsmY.

RsmY secondary structure

RNA folding algorithms [25] predicted that the 124 nucleotide (nt) long RsmY RNA [3] contains three stem-loop structures (see Fig. 3; I–III) with –GGA– motifs located in the loops as well as a rho-independent terminator (see Fig. 3; IV). The predicted RsmY secondary structure was experimentally tested by cleavage with the endoribonucleases T_1 and T_2, which cleave preferentially 3′ of unpaired guanine and adenine residues, respectively. Regions protected from cleavage by T_1 and T_2 in RsmY RNA were observed the stems of stem-loop structures I–III (Fig. 2A and B; lane 3). Stem-loop IV was likewise verified upon cleavage with RNase T2 (Fig. 2A, lane 3). According to these probing experiments three GGA-motifs are located in the loops of hairpins I–III (see Fig. 3). Thus, the structural pattern of *P. aeruginosa* RsmY RNA is similar to its *P. fluorescens* homologue [7], but the number of stem-loops and exposed –GGA– motifs varies.

RsmA and Hfq binding sites on RsmY RNA

It is known that Hfq binds preferentially to AU-rich regions adjacent to stem-loop structures [15,26,27]. Therefore RNase T_2, which cleaves 3′ of adenine residues, was used to map Hfq binding sites on RsmY RNA. Protection by Hfq against RNase T_2 cleavage was observed at sites A and B (Fig. 2A; lanes 4–6). As shown in Fig. 3, the Hfq binding sites on RsmY RNA agree with the criteria for Hfq binding sites; in that they are AU-rich and are overlapping with or adjacent to stem-loop structures. In addition, the mapping experiment agreed with the gel-mobility shift assays, in that RsmY RNA pre-bound to RsmA was able to bind two additional Hfq-hexamers (Fig. 1; B3 and B4).

As RsmA binding sites comprise -GGA-motifs RNase T_1, which cleaves 3′ of guanine residues, was used to reveal protection by RsmA of RsmY RNA. Protection by RsmA from Rnase T_1cleavage was observed at five positions in RsmY RNA (Fig. 2B; lanes 4–6, C–G). As anticipated from other studies on *P. fluorescens* [7,8] RsmY RNA, three RsmA binding sites encompass -GGA-motifs located in the loops of stem-loop structures (Fig. 3; C, F, and G). Two RsmA binding sites reside in single-stranded regions 5′ of hairpin II (Fig. 3; D and E), whereby one of them overlaps with Hfq binding site B (Fig. 3; D). In the gel-mobility shift assay two additional bands could be discerned after binding of RsmA to Hfq–RsmY complexes. Hfq was shown to have a higher affinity for RsmY than RsmA [21]. It is therefore, conceivable that binding of Hfq at positions A and B and/or structural changes imposed by Hfq binding interferes with RsmA binding at positions C, D, and E (Fig. 3).

Hfq binding blocks cleavage by RNase E

When compared with a *P. aeruginosa hfq*-strain, the stability and abundance of RsmY RNA is elevated in the corresponding wild-type strain [21]. As RNase E and Hfq binding sites can coincide [15], we tested whether Hfq can protect RsmY RNA from cleavage by the catalytic domain of *E. coli* RNase E *in vitro*. The N-terminal part of *E. coli* RNase E displays 68% identity with the N-terminal 540 amino acids of *P. aeruginosa* RNase E protein, and *E. coli* RNase E has been used to mimic *P. aeruginosa* mRNA processing [28]. Purified 5′-[^{32}P]-labelled RsmY RNA was subjected to *in vitro* cleavage by RNase E in the presence and absence of Hfq as indicated in Fig. 2A. Hfq-protected RsmY RNA from RNase E cleavage at five positions (Fig. 2A, arrows 1–5), at nucleotide position A10 in the single stranded region 5′ of stem-loop I, at positions A33 and A35 as well as at positions A39 and A40. *Escherichia coli* RNase E preferentially cleaves at the consensus sequence $(G,A)(C,A)N(G)(G,U,A)^{\Downarrow}(A,U)(C,U)N(C,A)(C,A)$ followed by stem-loop structures [29]. The five RNase E cleavage sites in RsmY sRNA do not completely share homology with the *E. coli* consensus sequence. Nevertheless, like in *E. coli* the RNase E cleavage sites in RsmY RNA are AU-rich, and are located in single-stranded regions adjacent to stem-loop structures (Fig. 3, arrows 1–5).

In summary, our results show that RsmA and Hfq can bind concomitantly to RsmY RNA (Fig. 1), and that binding of Hfq to RsmY can potentially protect RsmY from endonucleolytic cleavage by RNase E (Fig. 2A), which in turn can be reconciled with the observed increased stability of RsmY in the presence of Hfq [21]. Hfq has a higher affinity for RsmY than RsmA [21]. As binding of Hfq appears to interfere with RsmA binding (see Figs. 2 and 3), binding of Hfq to RsmY would result in a decreased capacity of RsmY to titrate RsmA. However, the 3-fold increase in the half-life of RsmY in the presence of Hfq [21], when compared to its absence, might compensate for the reduced titration capacity. Taken together, these results corroborated the hypothesis that binding of Hfq to RsmY does not eliminate the antagonizing function on RsmA [21]. Nevertheless, at present it cannot be excluded whether RsmY—in addition to its role in sequestration of RsmA—serves another role in gene expression. Since RsmZ, the only other ncRNA known in *P. aeruginosa* to titrate RsmA [2,3] did not interact with Hfq [21], and as many small regulatory RNAs require Hfq for translational silencing or activation of mRNAs [18], we are currently testing the possibility whether RsmY regulates translation of mRNA(s), i.e. whether it serves as well as a riboregulator.

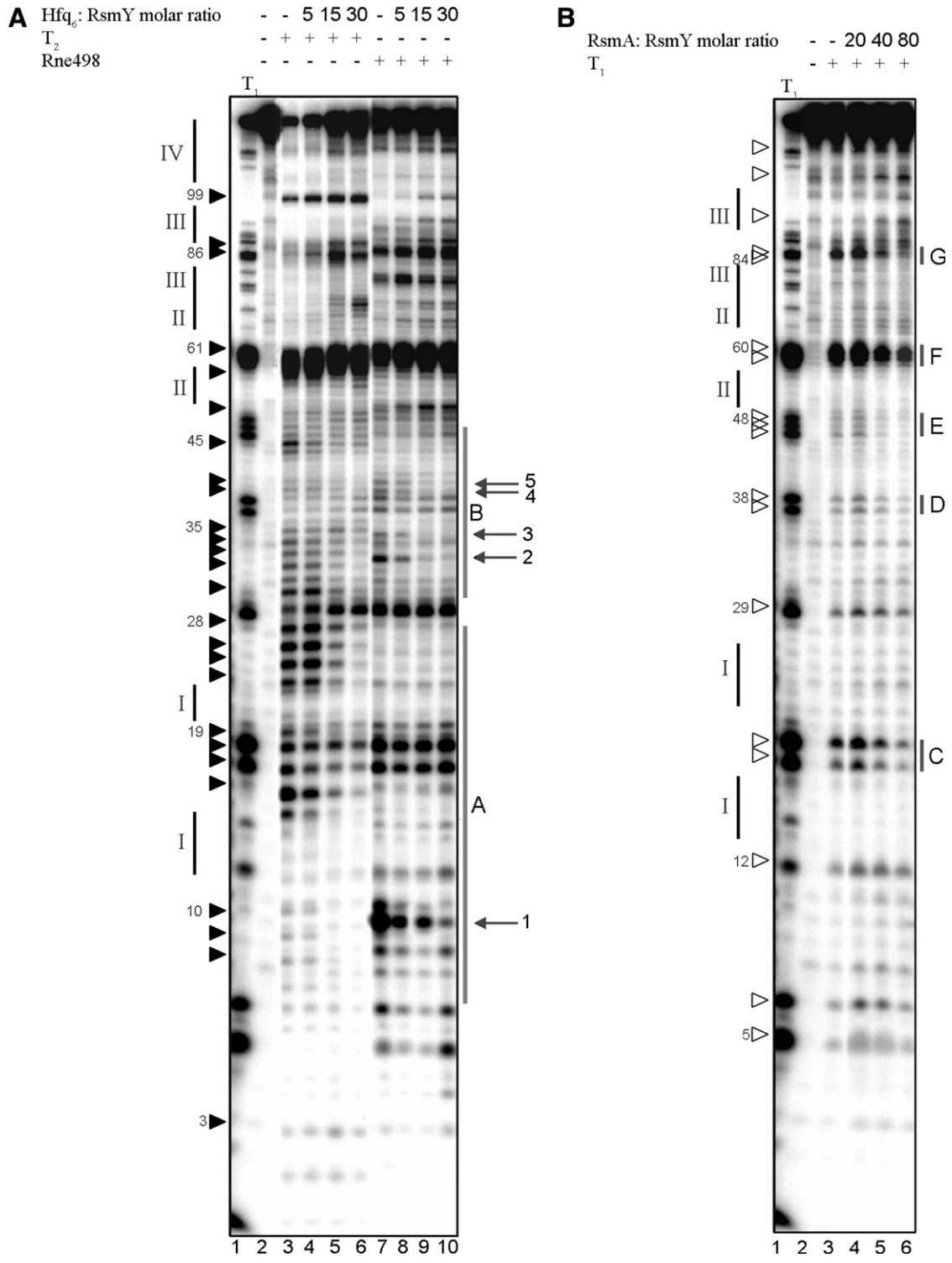


Fig. 2. Mapping of RsmA and Hfq_6 binding sites and RNase E cleavage sites in RsmY RNA. (A) 5′-[^{32}P]-labelled RsmY RNA (0.16 pmol) was incubated in the absence (lanes 2 and 3) or in the presence (lanes 4–6) of Hfq_6. The molar ratios of protein:RNA are indicated on top of the autoradiograph. *In vitro* RNase E cleavage of RsmY RNA was performed in the absence (lane 7) or in the presence (lanes 8–10) of Hfq. Filled arrowheads indicate RNase T_2 cleavage and their nt position in RsmY RNA. Bars at the left (I–IV) mark the position of stem-loop structures, where A's located in the stem are protected from RNase T_2 cleavage (lane 3). Bars A and B at the right indicate nt's protected from RNase T_2 cleavage by Hfq. The arrows numbered 1–5 at the right indicate RNase E cleavage sites in RsmY that are protected by Hfq. (B) 5′-[^{32}P]-labelled RsmY RNA (0.16 pmol) was incubated in the absence (lanes 2 and 3) or in the presence (lanes 4–6) of RsmA. The molar ratios of protein:RNA are indicated on top of the autoradiograph. Open arrowheads indicate RNase T_1 cleavage and their nt position in RsmY RNA. Bars at the left (I–IV) mark the position of stem-loop structures, where G's located in the stem are protected from RNase T_1 cleavage (lane 3). Bars C–G at the right indicate nt's protected from RNase T_1 cleavage by RsmA.

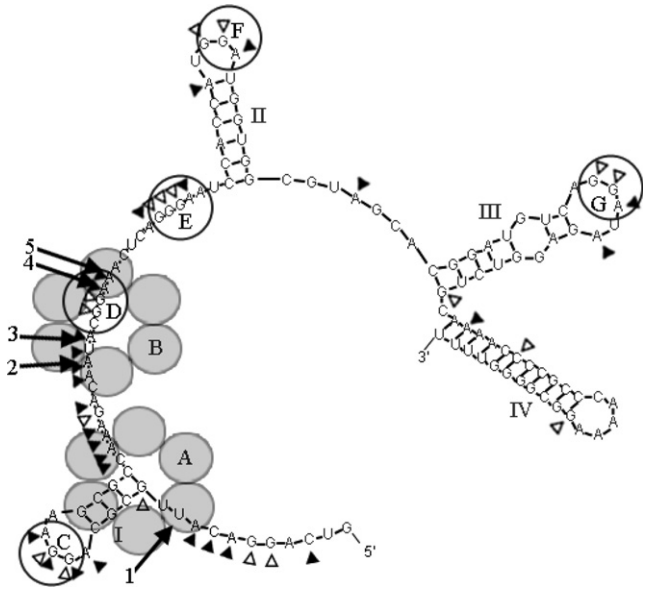


Fig. 3. RsmA (circles; C–G) and Hfq_6 (grey hexamers; A and B) binding sites and RNase E cleavage sites on RsmY RNA. The arrows numbered 1–5 indicate RNase E cleavage sites that are protected upon binding of Hfq to RsmY RNA. Roman numbers I–IV assign stem-loop structures. Filled arrowheads indicate RNase T_2 and open arrowheads RNase T_1 cleavage sites in RsmY RNA, in the absence of either Hfq or RsmA, respectively.

Acknowledgment

This work was supported by Grant F1715 from the Austrian Science Fund to U.B.

References

[1] M. Juhas, L. Eberl, B. Tümmler, Quorum sensing: the power of cooperation in the world of *Pseudomonas*, Environ. Microbiol. 7 (2005) 459–471.

[2] K. Heurlier, F. Williams, S. Heeb, C. Dormond, G. Pessi, D. Singer, M. Camara, P. Williams, D. Haas, Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1, J. Bacteriol. 186 (2004) 2936–2945.

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

[4] C. Valverde, S. Heeb, C. Keel, D. Haas, RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0, Mol. Microbiol. 50 (2003) 1361–1379.

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

[6] A.K. Dubey, C.S. Baker, T. Romeo, P. Babitzke, RNA sequence and secondary structure participate in high-affinity CsrA–RNA interaction, RNA 11 (2005) 1579–1587.

[7] C. Valverde, M. Lindell, E.G. Wagner, D. Haas, A repeated GGA motif is critical for the activity and stability of the riboregulator RsmY of *Pseudomonas fluorescens*, J. Biol. Chem. 279 (2004) 25066–25074.

[8] C. Reimmann, C. Valverde, E. Kay, D. Haas, Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0, J. Bacteriol. 187 (2005) 276–285.

[9] S. Heeb, K. Heurlier, C. Valverde, M. Camara, D. Haas, P. Williams, Post-transcriptional regulation in *Pseudomonas* spp. via the Gac/Rsm regulatory network, in: J.L. Ramos (Ed.), *Pseudomonas*, vol. 2, Kluwer Academic/Plenum Publishers, New York, NY, 2004, pp. 239–255.

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

[11] M.A. Schumacher, R.F. Pearson, T. Møller, P. Valentin-Hansen, R.G. Brennan, Structures of the pleiotropic translational regulator Hfq and an Hfq–RNA complex: a bacterial Sm-like protein, EMBO J. 21 (2002) 3546–3556.

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

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

[14] D.D. Sledjeski, C. Whitman, A. Zhang, Hfq is necessary for regulation by the untranslated RNA DsrA, J. Bacteriol. 183 (2001) 1997–2005.

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

[16] M. Folichon, V. Arluison, O. Pellegrini, E. Huntzinger, P. Regnier, E. Hajnsdorf, The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation, Nucleic Acids Res. 31 (2003) 7302–7310.

[17] S. Gottesman, The small RNA regulators of *Escherichia coli*: roles and mechanisms, Annu. Rev. Microbiol. 58 (2004) 303–328.

[18] N. Majdalani, C.K. Vanderpool, S. Gottesman, Bacterial small RNA regulators, Crit. Rev. Biochem. Mol. Biol. 40 (2005) 93–113.

[19] E. Massé, F.E. Escorcia, S. Gottesman, Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*, Genes Dev. 17 (2003) 2374–2383.

[20] E. Sonnleitner, S. Hagens, F. Rosenau, S. Wilhelm, A. Habel, K.E. Jager, U. Bläsi, Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1, Microb. Pathog. 35 (2003) 217–228.

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

[22] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, 1972.

[23] Y. Kuchino, S. Nishimura, Enzymatic RNA sequencing, Methods Enzymol. 180 (1989) 154–163.

[24] V.R. Kaberdin, A.P. Walsh, T. Jakobsen, K.J. McDowall, A. von Gabain, Enhanced cleavage of RNA mediated by an interaction between substrates and the arginine-rich domain of *E. coli* ribonuclease E, J. Mol. Biol. 301 (2000) 257–264.

[25] M. Zuker, Mfold web server for nucleic acid folding and hybridization prediction, Nucleic Acids Res. 31 (2003) 3406–3415.

[26] C.C. Brescia, P.J. Mikulecky, A.L. Feig, D.D. Sledjeski, Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure, RNA 9 (2003) 33–43.

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

[28] M. Gamper, D. Haas, Processing of the *Pseudomonas arcDABC* mRNA requires functional RNase E in *Escherichia coli*, Gene 129 (1993) 119–122.

[29] V.R. Kaberdin, Probing the substrate specificity of *Escherichia coli* RNase E using a novel oligonucleotide-based assay, Nucleic Acids Res. 31 (2003) 4710–4716.

[30] B.W. Holloway, V. Krishnapillai, A.F. Morgan, Chromosomal genetics of *Pseudomonas*, Microbiol. Rev. 43 (1979) 73–102.

CURRICULUM VITAE

Persönliche Daten

Name	Theresa Sorger-Domenigg
Anschrift	Schegargasse 18/12a
	1190 Wien
	Österreich
geboren	3.12.1979 in Graz
Staatsbürgerschaft	Österreich

Schulbildung

1998	Reifeprüfung
1990-1998	Gymnasium Sacré Coeur, Graz
1986-1990	Volkschule Neubaugasse, Graz

Studium

2007	einmonatiger Forschungsaufenthalt im Rahmen der Dissertation im Labor von Prof. Dr. Dieter Haas, Université de Lausanne, Département de microbiologie fondamentale, Schweiz
2005-2010	Dissertation am Department für Mikrobiologie, Immunbiologie und Genetik der Universität Wien ; Titel: „Novel *Pseudomonas aeruginosa* small regulatory RNAs“; Betreuer: Univ. Prof. Dr. Udo Bläsi
2004-2005	Diplomarbeit am Department für Mikrobiologie, Immunbiologie und Genetik der Universität Wien; Titel: „Interaction of Hfq with RsmY and its implications for quorum sensing in *Pseudomonas aeruginosa*“; Betreuer: Univ. Prof. Dr. Udo Bläsi
2001-2005	Universität Wien; Studienzweig: Mikrobiologie und Genetik
1998-2001	Universität Graz, Studienzweig: Mikrobiologie

DANKSAGUNG

Mein besonderer Dank gilt Univ. Prof. Dr. Udo Bläsi dafür, dass er mir die Möglichkeit gegeben hat diese Arbeit zu verfassen und mir stets ein sehr guter Betreuer war.

Ein großer Dank gebührt meinen Kollegen im Labor, die mir stets mit Rat und Tat zur Seite standen und eine angenehme Arbeitsatmosphäre geschaffen haben.

Ich möchte mich auch bei meiner Familie für die Unterstützung während meiner Studienzeit bedanken.