universität wien

DISSERTATION

Titel der Dissertation

„Characterisation of the regulatory RNA NalA in *Pseudomonas aeruginosa*“

Verfasserin

Mag. rer. nat. Alessandra Romeo

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 091 490
Dissertationsgebiet lt. Studienblatt:	Genetik-Mikrobiologie
Betreuerin / Betreuer:	Univ.-Prof. Dr. Udo Bläsi

„We do not know what we want and yet we are responsible for what we are - that is the fact“. (Jean-Paul Sartre)

„The only real progress lies in learning to be wrong all alone”. (Albert Camus)

TABLE OF CONTENTS

1. ZUSAMMENFASSUNG

Frei lebende oder endosymbiotischen Bakterien spielen eine zentrale Rolle im Kreislauf der verschiedenen Formen von Stickstoff, von anorganischem zu organischem Stickstoff und umgekehrt. Insbesondere wurde die Assimilation von Nitrat in komplexe Makromoleküle in *Klebsiella* spp, *Bacillus subtilis* und *Azotobacter vinelandii* ausgiebig untersucht. Im Gegensatz dazu haben sich Studien der Nitrat Nutzung in *Pseudomonas aeruginosa*, einem opportunistischen Krankheitserreger von Menschen, vor allem auf die Nitrat-Atmung konzentriert, die typischerweise in Biofilmpopulationen bei einer Infektion der Lungen von Mukoviszidose-Patienten beobachtet werden. Die *P. aeruginosa* Biofilme sind bekannt für ihre Resistenz gegen die Abwehrmechanismen des Wirts und antibakteriellen Behandlungen, was zu einer schlechten Prognose bei chronisch infizierten Patienten führt.

Die Auswirkungen kleiner regulatorischer RNAs (sRNAs) zum Anpassen bakterieller Genexpression wurde intensiv untersucht. Ein bioinformatischer Ansatz, um intergenische Regionen von *Pseudomonas aeruginosa* O1 (PAO1) für kleine RNAs zu analysieren, ergab ein putatives RNA-Gen upstream des Operon *nirBD*-PA1779-*cobA,* welches für die Nitratassimilation codiert. Diese RNA, NalA (Nitrate Assimilation leader A) genannt, steht für die Leader-RNA des *nirBD*-PA1779-*cobA* Operon. Es wurde gezeigt, dass die NalA Transkription, σ^{54} und NtrC abhängig ist. Ein PAO1 *nalA* Deletionsstamm und ein Stamm mit einer Deletion im ORF PA1785 kann nicht auf Nitrat wachsen. PA1785 wurde als Homolog des *Azotobacter vinelandii nasT* Gens identifiziert, dessen Produkt für die Transkription des *A. vinelandii* Nitrit / Nitrat-Reduktase-Operons gebraucht wird. Zusammengefasst zeigen diese Studien, dass transkriptionale Anti-Termination der Leader-RNA NalA für die Expression des Nitratassimilation Operons von PAO1 erforderlich ist, und dass dieser Prozess durch konservierte Funktionen in PAO1 und *A. vinelandii* reguliert wird.

Der zweite Teil der Studie beschäftigt sich mit der Frage, ob NalA die Regulation von Genen in *trans* vermitteln kann. Eine Transkriptomanalyse in PAO1 wurde durchgeführt, um Veränderungen in der Genexpression nach einer NalA Induktion nachzuweisen. Diese Analyse zeigte, verringerte Levels von Genen die mit der Typ IV-Pili Biogenese zusammenhängen, während die Levels der Gene, die an der oxidativen Resistenz und Gene, die im Zink Stoffwechsel beteiligt sind erhöht waren. Um den Einfluss von NalA auf die Expression des Typ IVb Pilin FLP-Gens und des *fimUpilVWXY1* Operon, das für Typ IVa Pili Biogenese erforderlich ist zu validieren, wurden Motilitäts und Biofilm-Assays durchgeführt. Darüber hinaus wurde die Expression des besonders hoch induzierten *dksA2* Gens nach NalA Induktion durch die Verwendung einer transkriptionalen p*dksA2-lacZ*-Reportergen-Fusion untersucht. Im Widerspruch zu den Microarray-Daten, hatte NalA keinen Einfluss auf PAO1 Motilität und Bildung von Biofilmen, und der Anstieg der mRNA-Levels von *dksA2* konnte nicht in Zusammenhang mit regulatorischen Ereignissen auf der Transkriptionsebene gebracht werden.

2. SUMMARY

Free-living or endosymbiotic Bacteria have a central role in cycling of the diverse forms of nitrogen, from inorganic to organic and *vice-versa*. In particular, the assimilation of nitrate into complex macromolecules has been extensively studied in *Klebsiella* spp., *Bacillus subtilis* and *Azotobacter vinelandii*. In contrast, studies of nitrate utilisation in the opportunistic human pathogen *Pseudomonas aeruginosa* have mainly focused on nitrate respiration, typically observed in biofilm populations infecting the lungs of cystic fibrosis patients. *P. aeruginosa* biofilms are renowned for their resistance against host defences and antibacterial treatments, leading to poor prognosis in chronically infected patients.

The impact of small regulatory RNAs (sRNAs) on adjusting bacterial gene expression has been intensively studied. Bioinformatic approaches employed to analyse intergenic regions of *Pseudomonas aeruginosa* O1 (PAO1) for small RNAs revealed a putative RNA gene encoded upstream of the nitrate assimilation operon *nirBD-PA1779-cobA.* This RNA, termed NalA (*n*itrogen *a*ssimilation *l*eader A), represents the leader RNA of the *nirBD-PA1779-cobA* operon. *NalA* transcription is shown to be σ^{54} and NtrC dependent. A PAO1 *nalA* deletion strain and a strain bearing a deletion in ORF PA1785 failed to grow on nitrate. PA1785 was identified as a homologue of the *Azotobacter vinelandii nasT* gene, the product of which is required for transcription of the *A. vinelandii* nitrite/nitrate reductase operon. Collectively these studies revealed that transcriptional antitermination of the leader RNA NalA is required for expression of the PAO1 nitrate assimilation operon, and that this process is governed by conserved functions in PAO1 and *A. vinelandii*.

The second part of the study addressed the question whether NalA acts in *trans* by mediating regulation of genes. A transcriptome analysis in PAO1 was performed to detect

changes in gene expression upon NalA induction. This analysis revealed reduced levels of genes pertaining to type IV pili biogenesis, whereas the levels of genes encoding oxidative resistance functions and zinc-related metabolism were increased. To validate the influence of NalA on expression of the type IVb pilin *flp* gene and of the *fimUpilVWXY1* operon required for type IVa pili biogenesis, motility and biofilm assays were performed. In addition, the expression of the most notably induced *dksA2* gene was studied following NalA induction by using a transcriptional p*dksA2-lacZ* reporter gene fusion. At variance with the microarray data, NalA had no influence on PAO1 motility and biofilm formation, and the increase in *dksA2* mRNA levels could not be attributed to regulatory events operating at the transcriptional level.

3. INTRODUCTION

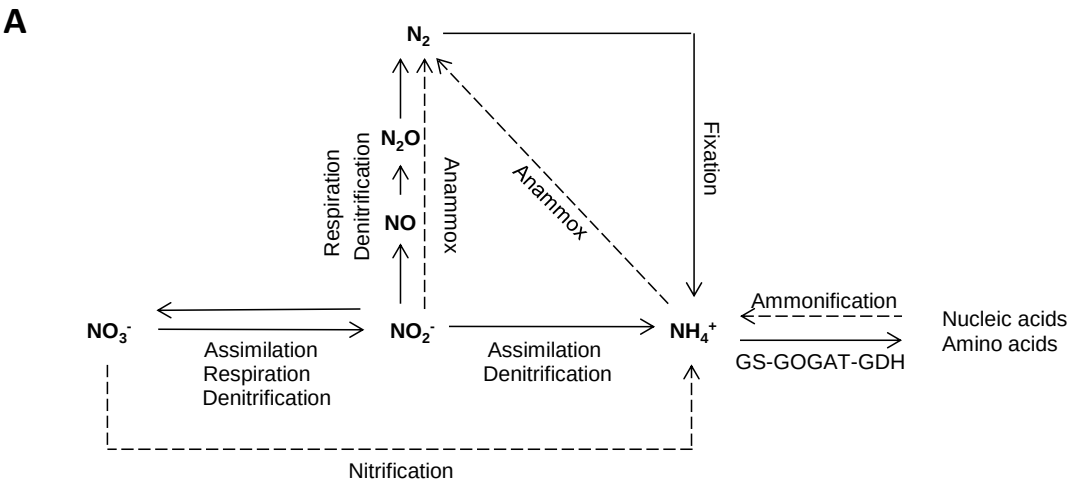
3.1. From organic to inorganic nitrogen and back: brief overview of the nitrogen cycle

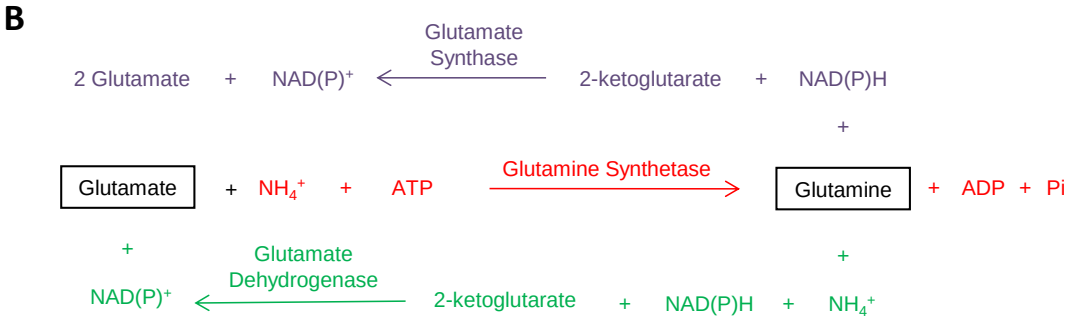
The nitrogen cycle (Figure 1A) represents the inter-conversion between various oxidation states of nitrogen (from N^{+5} to N^{-3}), changing from gaseous dinitrogen (N_2) or ammonia (NH_3) to ammonium (NH_4^+), nitrate (NO_3^-) or nitrite (NO_2^-) (Richardson & Watmough, 1999). In order to be incorporated into organic matter, such as nucleic acids and proteins, nitrogen is reduced to NH_4^+ through nitrogen fixation (Stewart, 1973; Kim & Rees, 1994) or nitrate assimilation/respiration (Lin & Stewart, 1998), usually achieved by free-living or endosymbiotic prokaryotes (Newton, 2007) and archaea (Cabello *et al.*, 2004). Conversely, NH_4^+ is regenerated from amino acid catabolism and decomposition during ammonification and further oxidized to NO_2^- and NO_3^- by nitrification (Klotz & Stein, 2008), and finally back to N_2 through anaerobic denitrification (Einsle & Kroneck, 2004). Although the direct oxidation of NH_4^+ to N_2, which occurs in the oceans during anaerobic ammonium oxidation (anammox), represents the main atmospheric nitrogen regeneration reaction, the mechanisms involved remain elusive (Thamdrup & Dalsgaard, 2002; Hu *et al.*, 2012).

3.2. The glutamate and glutamine checkpoints for intracellular nitrogen availability

3.2.1. The glutamine and glutamate synthesis pathways

The assimilated NH_4^+ is subsequently incorporated into glutamate and/or glutamine, the major donors for biosynthetic reactions (Figure 1B).





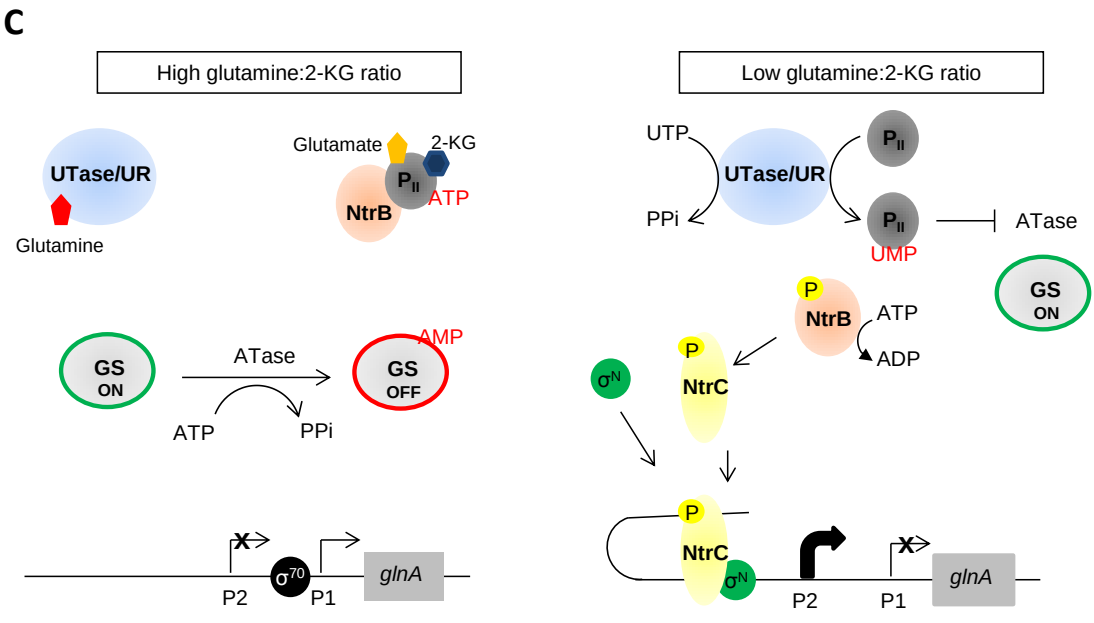


Figure 1. Nitrogen cycle and regulation in Bacteria. (A) Overview of inorganic nitrogen conversions. Arrows indicate reduction reactions, whereas dashed arrows designate oxidative reactions. (B) The GS/GOGAT pathway. The reaction accomplished by the glutamine synthase (GS) is marked in red, while glutamate synthesis through glutamate synthetase (GOGAT) or glutamate dehydrogenase (GSH) are in grren and blue, respectively. (C) The Ntr system for nitrogen assimilation control. See text for details. 2-KG: 2-ketoglutarate; ATase: adenyltransferase. P1: *glnA* promoter 1; P2: *glnA* promoter 2.

Glutamine is synthetized from glutamate, NH_4^+ and ATP by the glutamine synthetase enzyme (GS). Depending on the type of post-translational modification, four main forms of GS are known to date (Woods & Reid, 1993). The GSI enzyme is a heat stable 55 kDa dodecameric enzyme (Yamashita *et al.*, 1989), whose activity is inactivated through adenylation of a tyrosin group by a specific adenylyltransferase (ATase, Figure 1C) in response to intracellular nitrogen availability (Kustu *et al.*, 1984; van Heeswijk *et al.*, 1993; Woods & Reid, 1993). Synthesis of glutamate is carried out either by the glutamate synthase (GOGAT) from glutamine, 2-ketoglutarate and NAD(P)H (van den Heuvel *et al.*, 2004; Suzuki & Knaff, 2005), or by the glutamate dehydrogenase (GDH) by adding NH_4^+ to a 2-ketoglutarate backbone, using NAD(P)H as a proton donor (Hudson & Daniel, 1993).

3.2.2. Regulation of ammonium assimilation through the Ntr system

The intracellular abundance of nitrogen is sensed by the glutamine:2-ketoglutarate ratio. Nitrogen limitation lowers this ratio, thereby activating the Ntr system for nitrogen control (Engleman & Francis, 1978). The uridydyltrasferase/uridydyl-removing enzyme (UTase/UR) and the P_{II} protein sense any variations of this ratio, possibly through direct binding of glutamine/ATP and 2-ketoglutarate/glutamate, respectively (Kamberov *et al.*, 1995; Jiang *et al.*, 1998). Any decrease in the availability of ligands determines conformational changes in both proteins, so that the UTase/UR transfers a UMP group on residue Tyr-51 of P_{II} (Rhee, 1984; Son & Rhee, 1987). Uridylylated P_{II} protein stimulates de-adenylation and activation of the GS enzyme (Brown *et al.*, 1971; Mangum *et al.*, 1973; Adler *et al.*, 1975). The UTase/UR and P_{II} proteins further control downstream gene expression through the two-component system NtrBC, whereby NtrB acts as sensor protein while NtrC is the DNA-binding response regulator (Contreras & Drummond, 1988; Stock *et al.*, 1989; Figure 1C).

The NtrB sensor protein possesses both, a kinase and a phosphatase activity (Keener & Kustu, 1988). At high glutamine:2-ketoglutarate ratios, the P_{II} protein bound to NtrB stimulates its phosphatase activity. Both NtrB and NtrC are de-phosphorylated, and consequently inactivated in the presence of sufficient amounts of nitrogen (Figure 1C). In contrast, conformational changes in P_{II}-UMP prevent binding to NtrB, thus stimulating NtrB kinase activity. In turn, NtrB phosphorylates the His-139 residue on both of its own subunits (Ninfa & Bennett, 1991; Ninfa *et al.*, 1993), as well as on Asp-54 of NtrC (Moreno *et al.*, 1992). While not required for binding to DNA, phosphorylation of NtrC enables transcriptional activation of target genes (Weiss *et al.*, 1992; Mettke *et al.*, 1995), mostly in combination with σ^N, also termed σ^{54}. Binding of the σ^N-RNA polymerase (RNAP) holoenzyme to the promoter forms a transcriptionally inactive closed complex that requires the ATPase activity of phosphorylated NtrC for open complex formation (Weiss *et al.*, 1991; Austin & Dixon, 1992; Wedel & Kustu, 1995).

The *glnA* gene encoding the glutamate synthetase is among the genes regulated by the NtrBC two-component system. Driven by two distinct promoters (Dixon, 1984), *glnA* is constitutively expressed through its σ^{70}-dependent P1 promoter. Upon nitrogen limitation, phosphorylated NtrC and σ^{54}-RNAP mediate strong promoter P2-dependent expression of *glnA*. Expression from P2 inhibits transcription from P1; the activity of the two promoters is thus mutually exclusive (Reitzei & Magasanik, 1983; Ueno-Nishio *et al.*, 1984; MacFarlane & Merrick, 1985). Lastly, NtrBC are able to induce the expression of their own genes *ntrBC*, located downstream of, but transcriptionally independent from *glnA* (MacFarlane & Merrick, 1985).

3.3. Aerobic reduction of nitrate by Bacteria

3.3.1. Assimilatory nitrate/nitrite reductases

The reduction of nitrate to nitrite is the starting reaction for respiratory, dissimilatory and assimilatory nitrate utilization. The reaction occurs in the cytoplasm (Moreno-Vivián *et al.*, 1999). Two main nitrate transport have been characterized (Figure 2A), one of which is an ATP-binding cassette (ABC-type), exemplified by the *Klebsiella oxytoca* (*pneumoniae*) NasFED transport system (Figure 2A). Nitrate from the periplasm is bound to the NasF protein and is brought into the cytoplasm through the transmembrane protein NasED, where the ATPase function of the cytoplasmic homodimer NasD enables active transportation (Wu & Stewart, 1998). *A. vinelandii* (Setubal *et al.*, 2009), *Pseudomonas aeruginosa* (Winsor *et al.*, 2011) and *Bacillus subtilis* (Ogawa *et al.*, 1995) employ a type I NarK-like transporter for nitrate/proton symporter (Figure 2B). An integral membrane protein takes advantage of the proton electrochemical gradient in the periplasmic space to simultaneously transfer nitrate and protons to the cytoplasm (Rowe *et al.*, 1994).

Despite requiring common enzymes for the biosynthesis of the central molybdenum-containing cofactors (Noriega *et al.*, 2005), the three pathways employ functionally distinct nitrate reductases. As such, the assimilatory nitrate reductase Nas in the enteric bacterium *Klebsiella* (Figure 2B) is a heterodimer, whereby the larger subunit contains the molybdenum and molybdopterin-guanine dinucleotide (Mo-*bis*MGD) active site, where the reduction of nitrate to nitrite occurs. The required electrons and protons are donated by NAD(P)H to the FAD-containing small subunit. The protons and electrons are then channelled towards the active site through the large subunit iron-sulphur centres (one [4Fe-4S] and one [2Fe-2S];

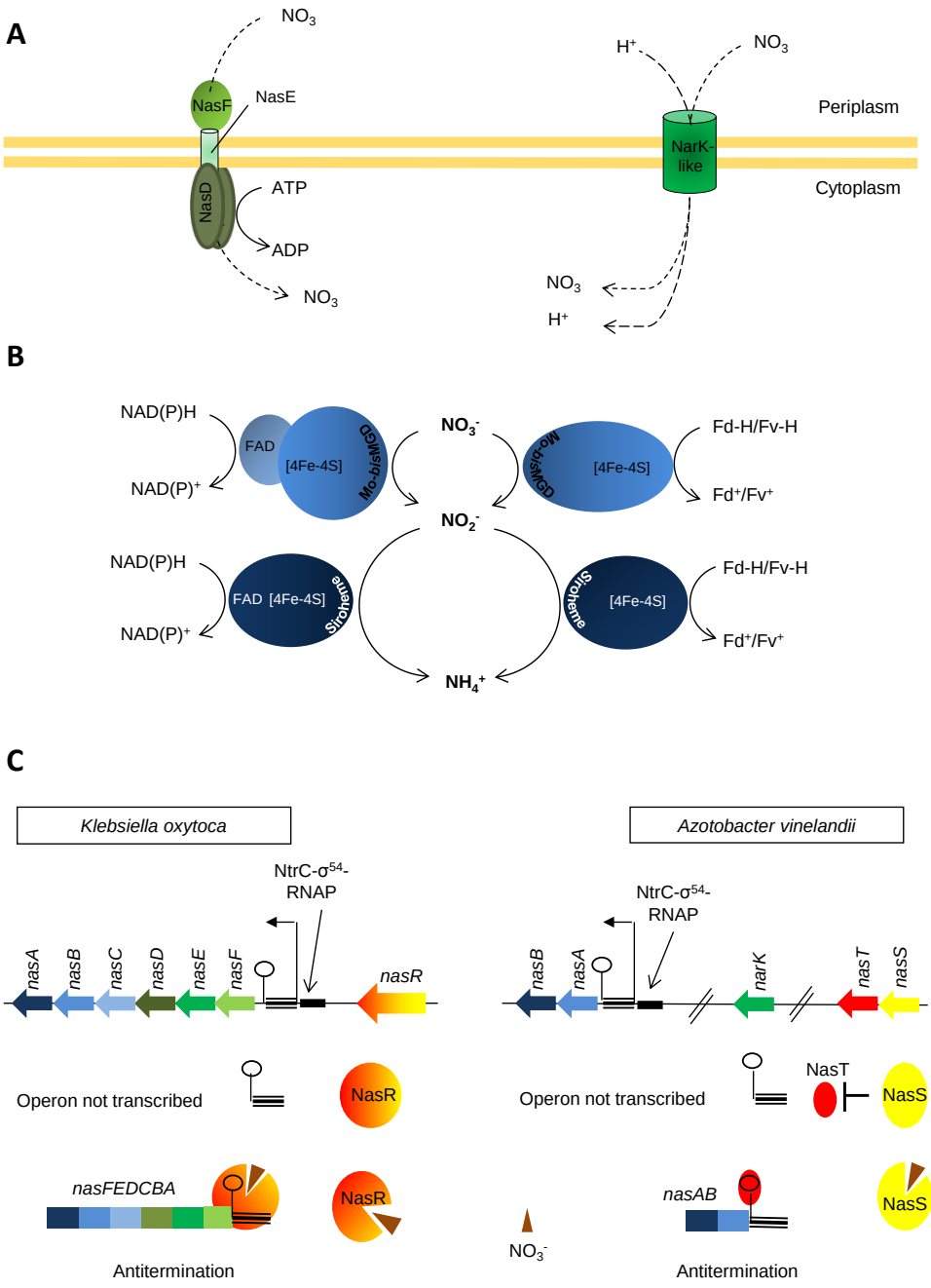


Figure 2. Nitrate assimilation in Bacteria. (A) Nitrate is brought into the cytoplasm by specific transport systems. ATP-driven (left) and NarK1-like proton:nitrate symporter (right). (B) Schematic view of the assimilatory nitrate (blue) and nitrite (dark blue) reductases. Left, *K. oxytoca* NAD(P)H-dependent heterodimeric nitrate and monomeric nitrite reductases; Right, Cyanobacterial and *A. vinelandii* ferredoxin (Fd) or flavodoxin (Fv) dependent monomeric nitrate and nitrite reductases. The figure was modified from Moreno-Vivián *et al.*, 1999. (C) Dual nitrogen- and nitrate-dependent regulation of the assimilatory nitrate/nitrite reductase operons. ORFs encoding functional classes of proteins are color coded: green arrows denote functions for nitrate transport, light blue arrows denote functions for the nitrate reductase, and dark blue arrows denote functions for the nitrite reductase. Expression of the operon is triggered by nitrogen limitation through NtrC and the σ^{54}-RNAP holoenzyme. Transcription is aborted in the absence of nitrate at the operon leader's rho-independent terminator. Transcription is resumed in the presence of nitrate by anti-termination, mediated by an RNA-binding protein. In *A. vinelandii* (right), sensing of nitrate and RNA antitermination are achieved by two proteins, NasS (yellow) and NasT (red), respectively. On the contrary, the *K. oxytoca* NasR protein (left) possesses both nitrate-sensing and RNA-binding properties (yellow-to-red protein). ↓: induction; ⊥: repression; See text for details.

Blasco *et al.*, 1997). In Cyanobacteria and in *Azotobacter vinelandii*, the Nas enzyme is monomeric and possesses one Mo-*bis*MGD centre and one [4Fe-4S] centre for electron and proton channelling (Rubio *et al.*, 1999; Rubio *et al.*, 2002). Flavodoxin or ferrodoxin are used as electron and proton donors in this case (Mikami & Ida, 1984).

In contrast to respiratory nitrite reduction, which occurs in the periplasmic space, the assimilatory reduction of nitrite to ammonium requires 6 protons, and is mediated by the assimilatory nitrite reductase (aNIR) in the cytoplasm (Moreno-Vivián *et al.*, 1999). All aNIR enzymes contain a siroheme active site and a [4Fe-4S] centre for proton channelling. However, while the NAD(P)H-dependent aNIR requires a FAD prosthetic group (Campbell & Kinghorn, 1990; Colandrene & Garrett, 1996), the ferrodoxin/flavodoxin-dependent aNIR does not (Campbell & Kinghorn, 1990; Luque *et al.*, 1993). The final product, ammonium is then directly used through the GS and GOGAT pathways.

3.3.2. Dual regulation of nitrate/nitrite assimilation

The assimilation of nitrate is repressed by ammonium through the Ntr control system. The genes required for both, nitrate and nitrite reduction, are usually part of a single operon, which is regulated by NtrBC and the σ^N (σ^{54})-RNAP holoenzyme. Expression of the operon is further regulated by a RNA leader, which possesses a strong, rho-independent terminator. In the absence of nitrate or nitrite, transcription of the operon is terminated at this terminator (Figure 2C). Transcription of the downstream genes requires anti-termination, mediated by a nitrate/nitrite sensing RNA-binding protein.

In *Klebsiella oxytoca* (*pneumoniae*), the NasR protein possesses both, a nitrate/nitrite sensing N-terminal domain, and an RNA-binding C-terminal domain (Goldman *et al.*, 1994). The binding of nitrate (or nitrite) to NasR results in a conformational change in the protein that enables binding to, and melting of, the *nasFEDCBA* operon leader (Lin & Stewart, 1996;

Chai & Stewart, 1998). In *Azotobacter vinelandii*, the nitrate/nitrite sensing and the antitermination functions are performed by separate proteins, NasS and NasT, respectively (Gutierrez *et al.*, 1995). In the absence of nitrate or nitrite, the NasS protein inhibits the RNA-binding activity of NasT. This functional inhibition is relieved by binding of nitrate/nitrite to NasS, consequently enabling NasT-mediated transcriptional antitermination of the *nasAB* operon (Gutierrez *et al.*, 1995). Although not experimentally validated, it is hypothesized that binding of nitrate affects the conformation of NasS, thus releasing a previously sequestered NasT (Gutierrez *et al.*, 1995; Lin & Stewart, 1998; Figure 2C).

3.4. The opportunistic human pathogen *Pseudomonas aeruginosa* (PAO1)

3.4.1. Specific adaptations enabling survival in the human host

In response to a complex environment, free-living Bacteria adapt to various stresses through an increase in genome size (Bentley & Parkhill, 2004). Conversely, the infection of a host usually leads to a drastic modification of gene expression (i.e., an increase in the number of pseudogenes), as the pathogen becomes specialized to less fluctuating conditions of the host environment (Ochman & Davalos, 2006).

With its relatively large genome (6,4 Mbp), the Gram-negative, γ-proteobacterium *Pseudomonas aeruginosa* possesses the largest number of regulatory two component systems of all sequenced Bacteria to date (Stover *et al.*, 2000; Rodrigue *et al.*, 2000), which appear to account for a high adaptability to different environments, including the human host. As an opportunistic human pathogen, infection by *P. aeruginosa* usually leads to poor prognosis in immunocompromised patients (Wood, 1976; Bodey *et al.*, 1983) and especially, in cystic fibrosis patients (Doring, 1993).

An acute infection by a pathogen requires the invasion and colonisation of the host, and is associated with local damage of host tissues. Acute infection by *P. aeruginosa* is achieved through expression of flagella required for motility (Feldman *et al.*, 1998), and by type III secretion systems (TTSS), required for host infection and virulence (Nicas & Iglewski, 1985; Fleiszig *et al.*, 1997; Filloux *et al.*, 2004).

The intrinsic antibiotic resistance of *P. aeruginosa* results from a general low permeability of the membrane, from the presence of various efflux pumps and from enzymatic modification of antibiotics (Breidenstein *et al.*, 2011; Moore & Flaws, 2011). Furthermore, the antibiotic resistance is enhanced by adaptive resistance mechanisms, which originate in the utilization of various antibiotics in hospitals (Alonso *et al.*, 1999). The uncontrolled utilisation of antibiotics consequently selects for panresistant strains, virtually impossible to eradicate (Rodríguez-Rojas *et al.*, 2011).

3.4.2. The switch from acute to chronic infections in *P. aeruginosa*

The transition from an acute to a chronic infection is regulated by two-component systems governing expression of specific acute and chronic infection genes (Goodman *et al.*, 2004; Furukawa *et al.*, 2006; Ventre *et al.*, 2007).

The translational repressor protein RsmA, belonging to the RsmA / CsrA family (regulator of secondary metabolism / carbon storage regulator), binds GGA motifs present in the leader of target mRNAs. As the location of this motif often coincides with the Shine and Dalgarno sequence, RsmA can impede translation initiation (Lapouge *et al.*, 2007; Schubert *et al.*, 2007). In this way RsmA acts as a negative regulator of secondary metabolites, virulence factors (e.g. siderophore, type VI secretion, extracellular enzymes) and carbon storage compounds (Burrowes *et al.*, 2006; Brencic & Lory, 2009). On the other hand RsmA is known to stimulate expression of genes required during acute infection (type III secretion, type IV pilus

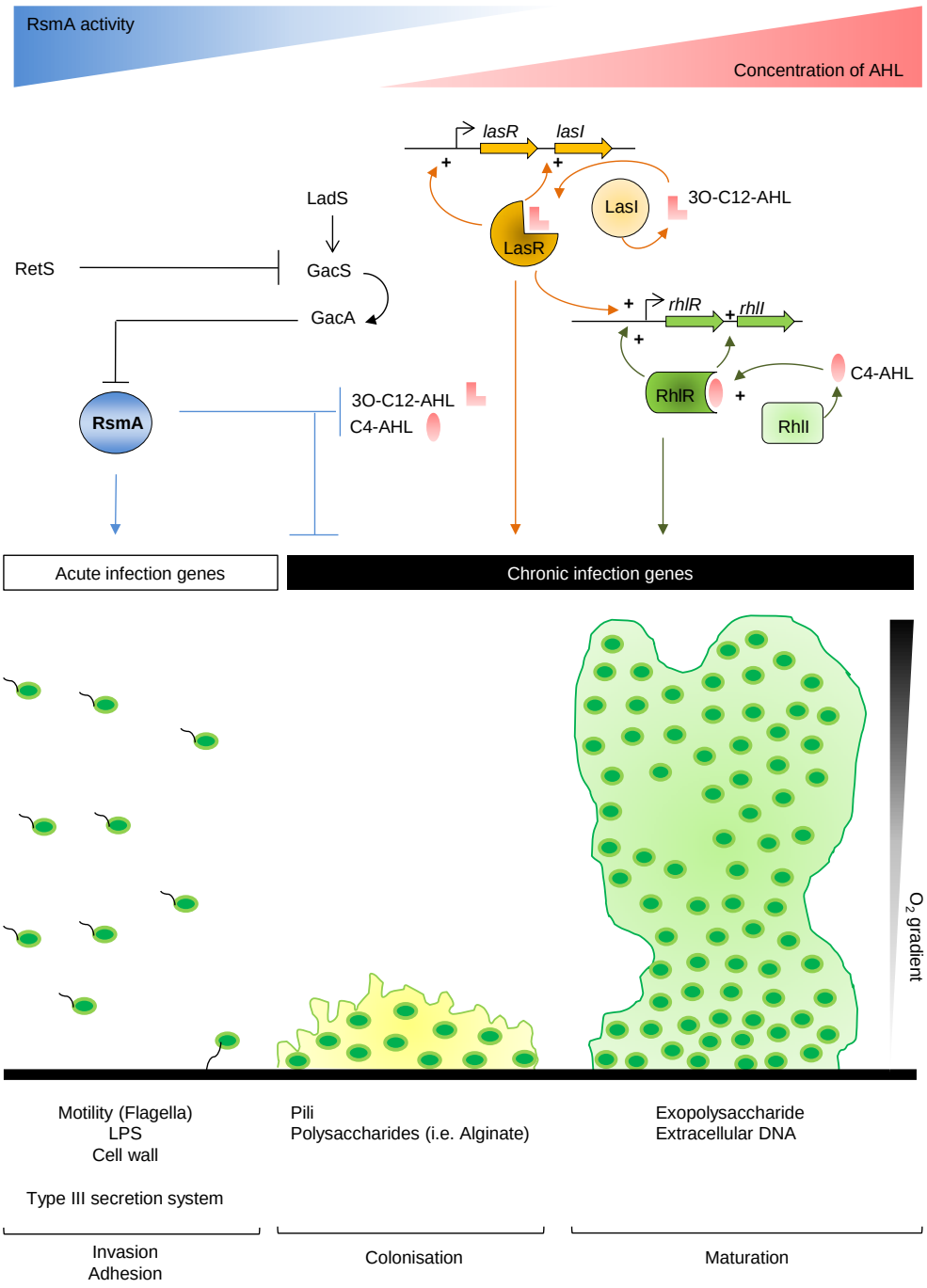


Figure 3. *Pseudomonas aeruginosa* regulatory network for acute/chronic infection and biofilm formation. Expression of acute infection genes is indirectly positively affected by the negative regulator RsmA, which represses translation of chronic infection genes (blue arrows). Under these conditions, the cells are mostly free-living and motile. RsmA activity is positively influenced by RetS signaling, while being repressed by the GacS/A signaling (through the sRNAs RsmY/Z, see part 3.5.1.1) and by LadS. Repression of RsmA leads to expression of chronic infection genes, as well as an increased synthesis of quorum sensing signaling molecules (and). The population-dependent and AHL-derived induction of *lasR* enables the downstream activation of RhlR signaling (orange and green arrows, respectively). The synthesis of further signaling molecules acts as positive feedback regulation for both *lasRI* and *rhlRI* operons. LasR and RhlR also stimulate expression of exoproducts like alginate, which determines the typical mucoid phenotype of nascent biofilms. Maturation of the biofilm occurs as the population grows embedded in the polysaccharide and DNA matrix, and reaches its characteristic mushroom-shaped structure. The list of phase-specific elements underneath the schematic biofilm maturation chart is only indicative and non-exhaustive. ↓: induction; ⊥: repression.

synthesis and flagellar motility). These positive effects are most likely achieved indirectly by RsmA-mediated interference with translation of regulatory factors (Brencic & Lory, 2009; Figure 3). More importantly, RsmA is a negative regulator of the quorum sensing circuitry. By repressing translation of the N-acyl-homoserine-lactones (AHL) synthases *lasI* and *rhlI*, RsmA restrains the production of the AHL, thereby repressing the quorum sensing circuitry (Pessi *et al.*, 2001; Figure 3).

The activity of RsmA is dependent on the GacS/A two component system (Reimmann *et al.*, 1997). In response to an unknown signal, the sensor GacS activates its cognate response regulator GacA through phosphorelay. The activated GacA indirectly represses RsmA activity through the small RNAs RsmY/Z, the mechanism of which is discussed below. The Gac/Rsm pathway is further regulated by two sensor kinases, RetS and LadS (Goodman *et al.*, 2004; Laskowski *et al.*, 2004; Laskowski & Kazmierczak, 2006; Ventre *et al.*, 2006; Goodman *et al.*, 2009). The dimeric RetS protein (Workentine *et al.*, 2009; Jing *et al.*, 2010) was shown to antagonize the GacS/A pathway by direct interaction with GacS (Goodman *et al.*, 2009). As a consequence, RetS negatively impacts in an indirect manner on genes required for chronic infection, whereas genes for acute infections are induced (Goodman *et al.*, 2004; Laskowski *et al.*, 2004; Laskowski & Kazmierczak, 2006; Ventre *et al.*, 2006). *P. aeruginosa retS* and *rsmA* mutant strains show similar phenotypes (Brencic & Lory, 2009); cell aggregation, over-production of extracellular polysaccharides, decreased expression of type III secretion functions and of type IV pili genes, the latter of which are required for twitching motility. On the other hand, LadS activation counteracts RetS signalling, promotes biofilm formation and represses type III secretion, thereby setting the stage for a chronic infection (Ventre *et al.*, 2006; Ventre *et al.*, 2007; Figure 3).

The formation of biofilm represents the landmark of a chronic infection. In biofilms the bacterial community is embedded in exopolysaccharides and in a nucleic acid matrix (Branda *et al.*, 2005; Figure 3) and successfully resists the host immune response as well as

antibiotic treatments (Mah & O'Toole, 2001; Matz & Kjelleberg, 2005; Weitere *et al.*, 2005; Hall-Stoodley & Stoodley, 2009; Høiby *et al.*, 2011). Alginate overproduction (Govan & Deretic, 1996), as well as outer membrane modification (Nakae, 1995; Sabra *et al.*, 2003; King *et al.*, 2009), further increase antibiotic tolerance. Adaptive modifications, which frequently affect regulatory genes (Oliver *et al.*, 2000; Smith *et al.*, 2006) can result in a mucoid phenotype (Mahenthiralingham *et al.*, 1994; Jain *et al.*, 2003; Li *et al.*, 2005). The initiation of biofilm formation is population-dependent (Davies *et al.*, 1998), and is mediated by the signalling secondary metabolites AHLs, also named autoinducers (Pearson *et al.*, 1994; Pearson *et al*., 1995; de Kievit, 2009). The two AHL quorum-sensing systems in *P. aeruginosa*, termed *las* and *rhl*, consist of an AHL synthase (LasI and RhlI, respectively) and of a transcriptional regulator (LasR and RhlR, respectively). The levels of AHL autoinducer increase in proportion to population growth. Upon reaching a threshold concentration, the LasR-specific N-(3-oxododecanoyl)-L-homoserine lactone (3-O-C12-HSL) binds to LasR and determines expression of downstream target genes, including *lasI* and *lasR* as positive feedback and signal amplifying loop, as well as *rhlR*. The increase in cell density also determines the accumulation of a second autoinducer, named N-butyryl-L-homoserine lactone (C4-HSL), which is RhlR-specific. Activation of RhlR upon binding of its ligand leads to transcriptional activation of RhlR-controlled genes, comprising *rhlI*. In turn, RhlI amplifies RhlR-dependent signalling by increasing the expression of its own operon, as well as inducing the expression of the stationary phase sigma factor σ^S, a master regulator of stress resistance and extracellular alginate, pyocyanin and exotoxin A production (Venturi, 2006; Schuster & Greenberg, 2006). Other signalling molecules, such as phenazine, induce biofilm formation in a quorum-sensing-independent fashion (Dietrich *et al.*, 2008; Figure 3).

3.4.3. Anaerobic nitrate respiration in the lungs of cystic fibrosis patients

Despite initial genetic studies aimed at dissecting the pathway for nitrate assimilation as a nutrient source in *Pseudomonas aeruginosa* (Sias & Ingraham, 1979; Jeter *et al.*, 1984), further studies were predominantly focused on nitrate respiration occurring in the lungs of cystic fibrosis (CF) patients (Kobayashi *et al.*, 2009; Høiby *et al.*, 2011).The lungs of CF patients are characterized by the presence of high density mucus due to the poor chloride transportation and consequent lack of water osmosis (Ratjen & Döring, 2003; Donaldson & Boucher, 2007). Moreover, the locally exacerbated innate immunity results in nitrate accumulation due to the activity of neutrophils (Downey & Elborn, 2000). While rich in various nutrients such as mucin and amino-acids (Barth & Pitt, 1996; Palmers *et al.*, 2005; Sriramulu *et al.*, 2005), *P. aeruginosa* grows typically in biofilms in CF lungs under oxygen limiting conditions (Worlitzch *et al.*, 2002; Yoon *et al.*, 2002) where nitrate is used as a final electron acceptor (Carlson & Ingraham, 1983; Davies *et al.*, 1989; Zumft, 1997).

The respiratory nitrate reduction occurs in the cytoplasm (Cabello *et al.*, 2004), and is achieved by the membrane-bound nitrate reductase complex NarGHI (Figure 4A). The active site of the catalytic subunit NarG contains a Mo-bisMGD centre as well as [3Fe-4S] and [4Fe-4S] iron-sulphur clusters for electron and proton channelling. The latter are provided by the menaquinone pool (MQH_2/MQ^+), and are transferred to the periplasmic space (Berks *et al.*, 1995; Philippot & Højberg, 1999) by the β-types heme centres of the membrane integral NarI subunit (Magalon *et al.*, 1998). The electrons and protons required for NarG-mediated reduction of nitrate (Gregory *et al.*, 2003) are brought back into the cytoplasm through the NarK1 proton:nitrate transporter, which also mediates nitrate transport in the cytoplasm (Berks *et al.*, 1995). The final product nitrite is then exported to the periplasmic space through a second transporter, the nitrate:nitrite antiporter NarK2 (Berks *et al.*, 1995). The accumulating nitrite is detoxified in the periplasmic space to nitric oxide, nitrous oxide and finally, to dinitrogen

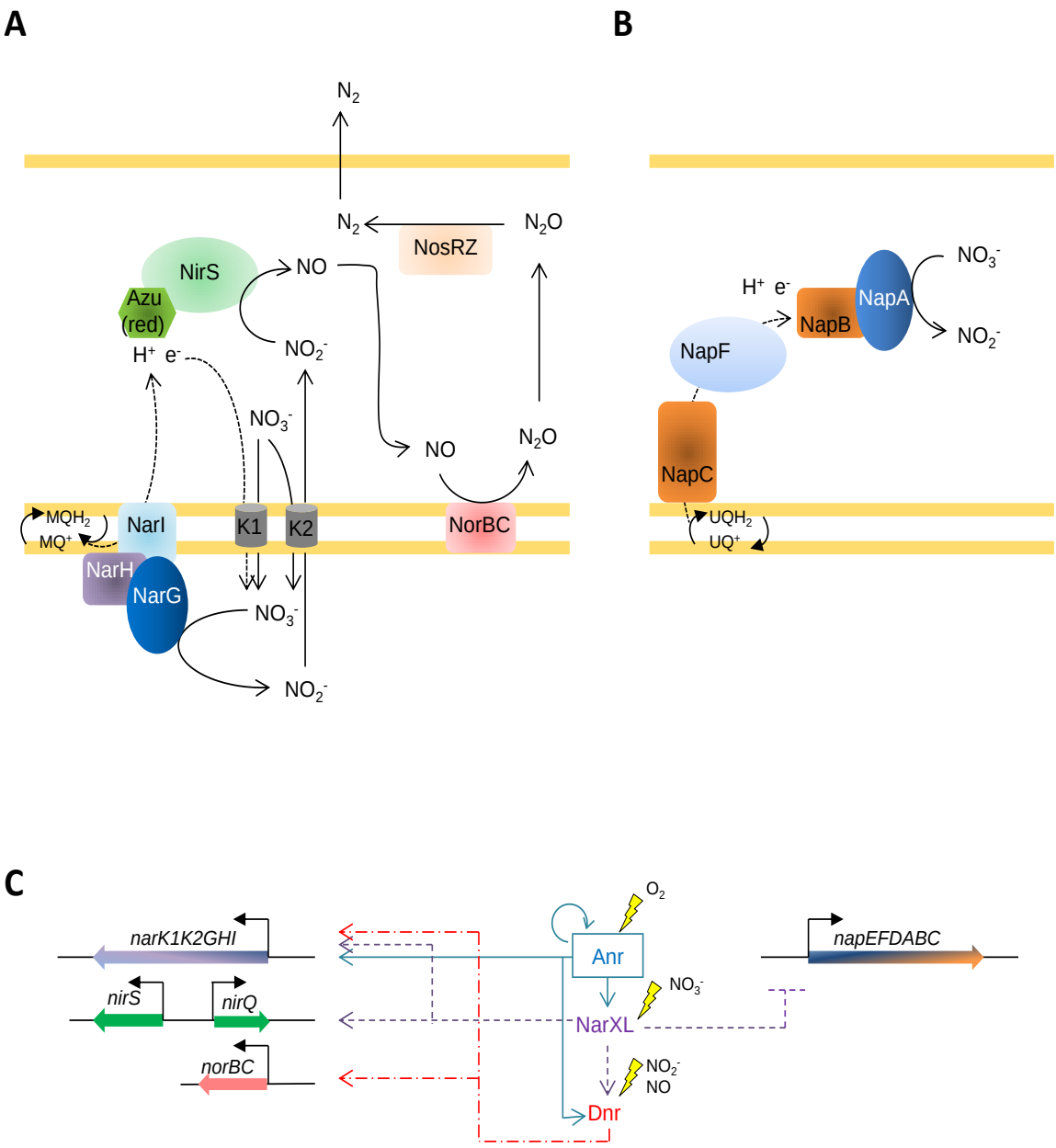


Figure 4. Nitrate respiration/dissimilation in *Pseudomonas aeruginosa* colonizing the lungs of CF patients. Schematic representation of the electron/proton transfer (dashed arrows) and reduction reactions (arrows) occurring during nitrate respiration (A) and periplasmic nitrate dissimilation (B). The required H^+/e^- are donated by MQH_2/MQ^+: menaquinone redox pool; UQH_2/UQ^+: ubiquinone redox pool. K1/K2: short for NarK1 and NarK2 nitrate transporters, respectively. (C) Oxygen-dependent regulatory network for expression of both, respiratory and dissimilatory nitrate reductase operons. ORFs encoding functional classes of proteins are color coded: blue arrows denote functions for the nitrate reductase, green arrows denote functions for nitrite reductase and pink arrow denotes functions for nitric oxide reductase. The *nap* operon is depicted as a blue-to-orange arrow. The genes affected by the transcriptional regulators Anr, NarXL two-component system and Dnr are indicated by light blue, dashed light purple and dashed red, respectively. ↓: induction; ⊥: repression; See text for details.

gas in three independent reactions accomplished by nitrite reductase NirS, nitric oxide reductase NorCB (Kawasaki *et al.*, 1997) and nitrous oxide reductase NosRZ (Zumft, 1997), re-

spectively. In addition, arginine fermentation (Vander Wauver *et al.*, 1984; Luthi *et al.*, 1990) and pyruvate fermentation (Eschbach *et al.*, 2004) represent two non-redox energy generating systems under anaerobic conditions (Platt *et al.*, 2008). However, both pathways poorly support growth (Eschbach *et al.*, 2004).

The dissimilatory periplasmic nitrate reductase (Figure 4B), a third, still poorly characterized pathway for microaerobic nitrate dissimilation, was detected in *Pseudomonas aeruginosa* biofilms (Beckmann *et al.*, 2005; Manos *et al.*, 2009). The periplasmic nitrate reductase NapAB heterodimer catalyses the reduction of nitrate through its Mo-*bis*MGD core in the periplasmic space (Flanagan *et al.*, 1999). The required electrons and protons are donated by the ubiquinone pool (UQH_2/UQ^+), then channelled to the cytochrome c-type protein NapC (Reyes *et al.*, 1996; Berks *et al.*, 1995), and finally transferred to the NapAB enzyme through the [4Fe-4S] centres of the NapF protein (Brondijk *et al.*, 2002; Olmo-Mira *et al.*, 2004; Nilavongse *et al.*, 2006). Although not employed to generate a proton motive force for energy synthesis (Potter *et al.*, 2001), the periplasmic nitrate reduction pathway was shown to dissipate an excess of reducing power (Reyes *et al.*, 1996; Potter *et al.*, 2001; Gavira *et al.*, 2002), as well as catalyse the first step of denitrification during transition from aerobic to anaerobic conditions (Bedzyk *et al.*, 1999). Depending on the oxygen tension within the three dimensional structure of the biofilm bacterial community (Xu *et al.*, 1998; Werner *et al.*, 2004), the reduction of nitrate through the Nap enzymes could be necessary under microaerobic conditions.

Activation of the membrane-bound and periplasmic nitrate reduction and the two non-redox fermentative pathway are dependent on oxygen tension (Figure 4C). The sensor protein Anr (Ye *et al.*, 1995; Eschbach *et al.*, 2004) contains a [4Fe-4S] centre, which is inactivated in the presence of oxygen (Yoon *et al.*, 2007). Under anaerobic condition, Anr acts as a DNA-binding homodimer, and stimulates the expression of the arginine and pyruvate fermentative pathways, as well as the two-component system *narXL* and the *dnr* genes (Schreiber *et al.*,

2007). The nitrate-sensing NarX activates its cognate response regulator NarL through phosphorelay in the presence of nitrate, leading to the expression of the respiratory nitrate reduction operon *narK1K2GHJI* (Schreiber *et al.*, 2007). In addition, NarL further enhances expression of *dnr*, and conversely, represses both, the fermentative pathways (Benkert *et al.*, 2008) and the periplasmic nitrate reductase (Van Alst *et al.*, 2009) operons. Detoxification of nitrite requires the respiratory nitrite reductase NirS, the maturation of which is dependent on the expression of *nirQ*. The expression of *nirQ* requires both, Dnr and NarL (Arai *et al.*, 1994; Arai *et al.*, 1995; Hayashi *et al*., 1998). The presence of the highly reactive nitric oxide might lead to severe oxidative damage, especially through Fenton reactions involving Fe-S centres (Spiro, 2007; Pullan *et al.*, 2008). As a consequence, nitric oxide detoxification is performed by Dnr, which detects its production through conformational changes in response to nitric oxide binding to its ferrous heme centre (Giardina *et al.*, 2008; Giardina *et al.*, 2009; Castiglione *et al.*, 2009). The activated Dnr homodimer induces expression of the *norCB* and the *nosRZDFYL* operons, and further amplifies Anr and NarXL signalling (Scheiber *et al.*, 2007). Finally, the expression of the *nar*, *nir*, *nor* and *nos* operons is negatively regulated by the quorum sensing signalling network (Toyofuku *et al.*, 2007).

3.5. Small RNAs in PAO1

The majority of the studied small RNAs (sRNAs) appear to adjust bacterial physiology in response to environmental changes or stress conditions (Waters & Storz, 2009). Moreover, in several Bacteria including *P. aeruginosa* sRNAs are involved in the regulation of virulence genes (Kay *et al.*, 2006; Oglesby *et al.*, 2008). Three major classes of small regulatory RNAs are known. One class of sRNAs acts by base-pairing with mRNAs, affects their stability and / or their translational output, and often requires the RNA chaperone Hfq for function

(Gottesman, 2004; Kaberdin & Bläsi, 2006; Papenfort & Vogel, 2009). The second class of sRNAs interacts with proteins and modulates their activity (Kazantsev & Pace, 2006; Babitze & Romeo, 2007; Wassarman, 2007). Lastly, sRNAs can target DNA, as exemplified by the clustered, regularly interspaced, short palindromic repeats (CRISPR), an adaptable and inheritable defense system of prokaryotes that provides them with immunity against accessory genetic elements (van der Oost *et al.*, 2009; Al-Attar *et al.*, 2011). To date, CRISPR systems are detected in pathogenic *P. aeruginosa* strains (i.e. PA14) (Cady *et al.*, 2011), and despite recognising foreign DNA from bacteriophages as well as transposable elements (Mojica *et al.*, 2005; Cady & O'Toole, 2011), this does not lead to DNA degradation (Zegans *et al.*, 2009).

3.5.1. sRNAs acting by protein sequestration

3.5.1.1. RsmY/Z

As previously mentioned, the establishment of a chronic infection, the highest adaptation of pathogens to the host environment, relies on the Gac/Rsm system. In *P. aeruginosa* the function of RsmA is regulated by two functional redundant sRNAs, RsmY and RsmZ (Figure 5). Activation of the GacS/GacA (global activation) two-component system *via* phosphorelay between the sensor GacS and its cognate response regulator GacA results in transcription of these sRNAs (Brencic *et al.*, 2009; Goodman *et al.*, 2009). GacA-mediated induction of RsmY and RsmZ synthesis seems to require an evolutionary conserved upstream activating sequence (UAS) (Brencic *et al.*, 2009; Humair *et al.*, 2010). Although the sRNAs RsmY and RsmZ share a limited sequence conservation, they are structurally conserved and possess seven (RsmY) and eight (RsmZ) GGA motifs, respectively, located in single-stranded RNA regions, mostly in loops or between stem-loop structures (Heeb *et al.*, 2002; Valverde *et al.*, 2004; Kay *et al.*, 2005). These motifs allow RsmY and RsmZ to bind to and to sequester

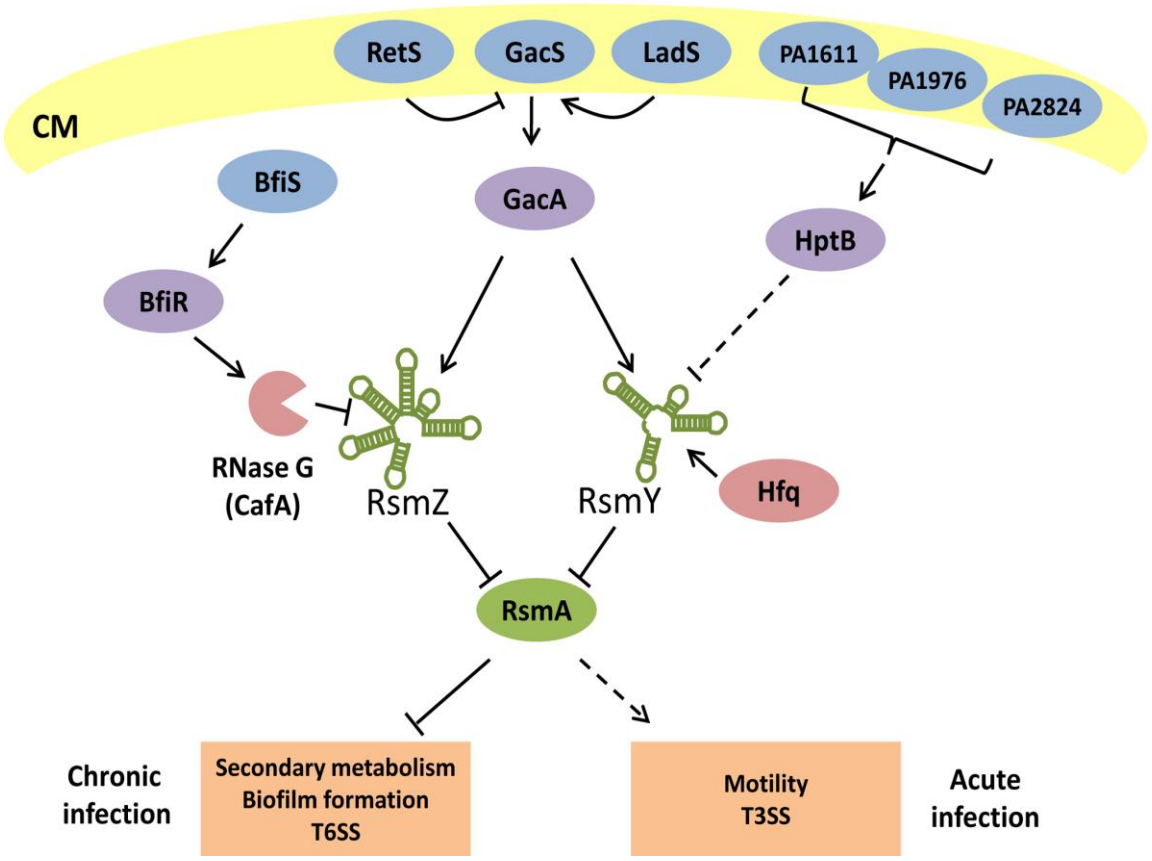


Figure 5. Gac/Rsm regulatory network in *P. aeruginosa*. The synthesis and stability of the RsmZ and RsmY RNAs depends on several two-component systems (sensors are shown in blue and response regulators in purple). Some regulation occurs on both sRNAs, e.g. transcriptional activation by GacA, whereas other control elements act only on one sRNA, e.g. CafA mediated degradation of RsmZ and Hfq-mediated stabilization of RsmY. This regulation allows the sRNAs to respond to different environmental signals. As they counteract the activity of the translational regulator RsmA by binding to and sequestering the protein, they mediate the switch between chronic and acute infection (see text). ↓: positive control; ⊥: negative control; dashed lines: indirect control.

RsmA (Valverde et al., 2004), which in turn leads to de-repression of RsmA target mRNAs (Pessi *et al.*, 2001; Heeb *et al.*, 2006; Kay *et al.*, 2006; Brencic & Lory, 2009). While the synthesis of RsmY and RsmA increases throughout growth, *rsmZ* expression is predominantly observed in late growth phase (Kay *et al.*, 2005). Moreover, RsmY is stabilized in *P. aeruginosa* by binding to the RNA chaperone Hfq (Sonnleitner *et al.*, 2006), or concomitantly to RsmA and Hfq, which appears to confer protection from RNase E (Sorger-Domenigg *et al.*, 2007). The abundance of RsmY and RsmZ is further modulated by a number of regulators. RsmY and RsmZ directly influence their own transcription through an unknown negative feedback mechanism (Ventre *et al.*, 2007; Lapouge *et al.*, 2008). Through possible physical

contacts with GacS (Workentine *et al.*, 2009), LadS is able to up-regulate *rsmZ*, thereby antagonizing RetS signalling (Kay *et al.*, 2006; Ventre *et al.*, 2006). The global regulators MvaT and MvaU (H-NS family) are able to silence *rsmZ* expression by binding the A+T-rich DNA segment upstream of *rsmZ* (Brencic *et al.*, 2009). Furthermore, the histidine phosphotransfer protein B (HptB) negatively impacts on *rsmY* expression as a result of a complex phosphorelay occurring between three sensor kinases (PA1611, PA1976 and PA2824) and HptB. Phosphorylated HptB then acts only on *rsmY*, possibly by transferring the phosphate to PA3347, a putative anti-anti-sigma factor (Hsu *et al.*, 2008; Bordi *et al.*, 2010). In addition, in *P. aeruginosa* biofilms the two component system BfiSR was shown to affect the abundance of RsmZ by activating transcription of *cafA*, encoding a protein with ribonuclease G activity that specifically cleaves RsmZ but not RsmY (Petrova & Sauer, 2010).

3.5.1.2. CrcZ

Carbon catabolite repression is used by Bacteria to catabolize substrates that enable optimal energy gain and growth. It is enacted in the presence of the preferred carbon source usually through repression of genes, the products of which are required for the utilization of less-preferred carbon sources (Görke & Stülke, 2008; Rojo, 2010). The RNA binding protein Crc (catabolite repression control protein, Figure 6) recognizes CA-rich sequences (CA-motifs) in the vicinity of the ribosome binding sites (Moreno *et al.*, 2007; Moreno *et al.*, 2009; Sonnleitner *et al.*, 2009). This results in translation inhibition of mRNAs that encode enzymes required for the utilization of less-preferred carbon sources (Moreno & Rojo, 2008; Moreno *et al.*, 2010), such as *amiE* mRNA encoding the aliphatic amidase (Smyth & Clarke, 1975; Sonnleitner *et al.*, 2009). On the basis of the knowledge gained on the above mentioned RsmA/Y/Z system, the Haas group searched for CA-motifs in intergenic regions potentially

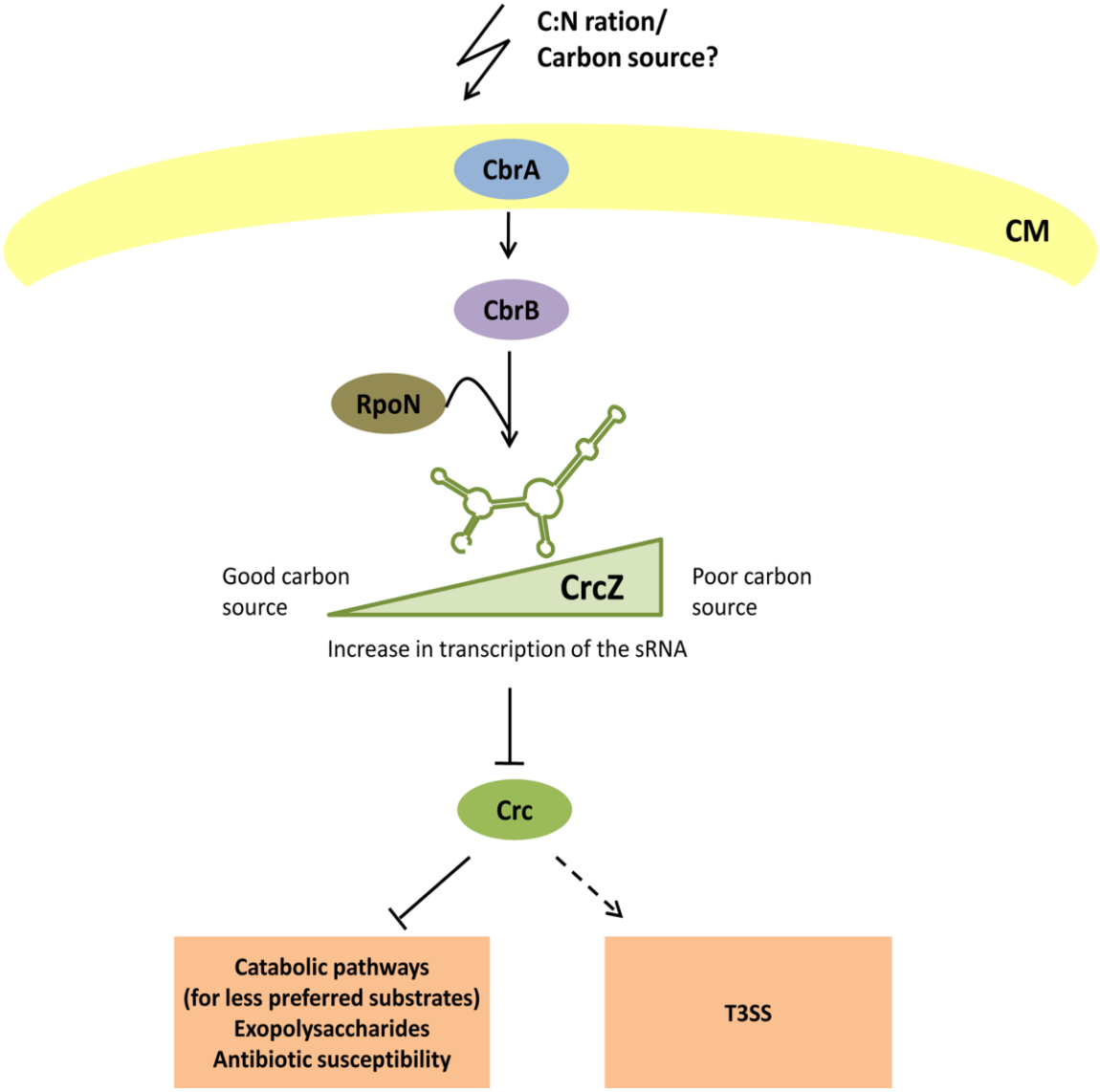


Figure 6. CrcZ-mediated regulation in *P. aeruginosa*. In the presence of a preferred carbon source (e.g. succinate), the CrcZ levels are low, whereas a poor carbon source (e.g. mannitol) leads to up-regulation of *crcZ*. The increased expression of *crcZ* is mediated by the activity of the two component system CbrAB and the sigma factor RpoN. When CrcZ is present, the sRNA binds to and sequesters the translational regulator Crc, which leads to activation of target genes. The respective enzymes / proteins are involved in catabolism of less preferred carbon sources, but also in exopolysaccharide production and antibiotic susceptibility. In contrast, genes implicated in the Type 3 secretion system (T3SS) are repressed in the absence of Crc, most likely by an indirect mechanism. ↓: positive control; ⊥: negative control; dashed lines: indirect control.

encoding sRNAs. These studies led to the discovery of the RNA CrcZ (Sonnleitner *et al.*, 2009; Figure 8). The 407 nt sRNA CrcZ comprises five CA-motifs and was shown to antagonize the function of Crc in catabolite repression. When *P. aeruginosa* grows on a less preferred carbon source such as mannitol or acetamide, CrcZ binds to and sequesters Crc, allow-

ing synthesis of the required catabolic enzymes for utilisation of these substrates (Sonnleitner *et al.*, 2009).

Interestingly, the discovery of *P. aeruginosa* sRNA CrcZ has linked the Crc function to the CbrA/B two-component system (catabolic regulation two-component system) (Nishijo *et al.*, 2001; Li & Lu, 2007; Sonnleitner *et al.*, 2009). The transcription of CrcZ is driven by CbrAB in a σ^{54}-dependent manner. The type of carbon source determines the activity of CbrAB, and thus the CrcZ level, which is low in the presence of a preferred carbon source, like succinate, and elevated in the presence of an intermediate substrate, like glucose, and high in the presence of a poor carbon source such as mannitol (Sonnleitner *et al.*, 2009). Similarly, the Crc levels can vary in a manner dependent on growth conditions, as observed in *P. putida* (Ruiz-Manzano *et al.*, 2005) and in *P. aeruginosa* (E. Sonnleitner, unpublished results). In addition, the CbrAB/Crc pathway is likely to impact on several other functions. For instance, Crc was shown to inhibit the synthesis of porins (Linares *et al.*, 2010), of the virulence factor pyocyanin and of exopolysaccharides. A *P. aeruginosa crc* mutant showed an increased sensitivity towards certain antibiotics and is impaired in twitching motility, type III secretion, and biofilm formation (O'Toole *et al.*, 2000; Linares *et al.*, 2010).

3.5.2. sRNA acting by a base-pairing mechanism

3.5.2.1. PrrF1/PrrF2

Being a cofactor of a large number of enzymes iron is pivotal for cellular metabolism as well as for colonization of mammalian hosts by bacterial pathogens. In contrast, under oxygen-rich conditions iron is a source of toxic radicals, which can damage cellular components. Homeostatic regulatory systems ensure the adaptation of *P. aeruginosa* to low-iron conditions

by increasing the production of siderophore-dependent iron uptake systems, in combination with iron storage proteins (i.e. bacterioferritin, *bfrB*) and of enzymes (e.g. catalase, *katA*, and iron-containing superoxide dismutase, *sodB*) that neutralize oxidative stress resulting from the toxic effects of excess iron). In most Gram-negative Bacteria the ferric uptake regulator Fur complexed with Fe^{2+} ions prevents transcription of genes involved in iron acquisition and of iron-regulated genes involved in virulence (Hantke, 2001). In *E. coli*, the Fur-repressed sRNA RhyB down-regulates the expression of genes encoding iron-containing proteins through base-pairing (Večerek *et al.*, 2003; Geissman & Touati, 2004).

A bioinformatics search for a functional ortholog of RhyB in *P. aeruginosa* revealed the sRNAs PrrF1 and PrrF2 (Wilderman *et al.*, 2004). These sRNAs are encoded in tandem, share 95% identity, and both genes are preceded by a Fur binding site (Figure 7). Consequently, the PrrF1 and PrrF2 sRNAs are synthesized during iron limitation, i.e. when Fur does not function as a repressor. A microarray analysis revealed a large number of candidate PrrF-regulated genes. Among them were several functions involved in iron storage and protection from oxidative stress. In analogy with RhyB, PrrF1 and PrrF2 are predicted to base-pair with the ribosome binding site of *sodB* mRNA, which appears to result in translational inhibition and consequently in degradation of the mRNA (Wilderman *et al.*, 2004; Oglesby *et al.*, 2008). However, the majority of PrrF-repressed genes appear to encode enzymes that participate in aerobic and anaerobic metabolism (Vasil, 2007; Oglesby *et al.*, 2008). Several of these candidate functions are involved in the tricarboxylic acid (TCA) cycle, e.g. the succinate dehydrogenase (*sdhCDAB*), the aconitases A and B and a putative isocitrate lyase, which are likewise repressed by RyhB in *E. coli* (Massé & Gottesman, 2002; Massé *et al.*, 2005). In support, the growth of a *P. aeruginosa* Δ*prrF1/prrF2* mutant was impaired under low-iron conditions and in minimal medium supplemented with succinate, presumably as a result of aberrant TCA cycle function (Vasil, 2007).

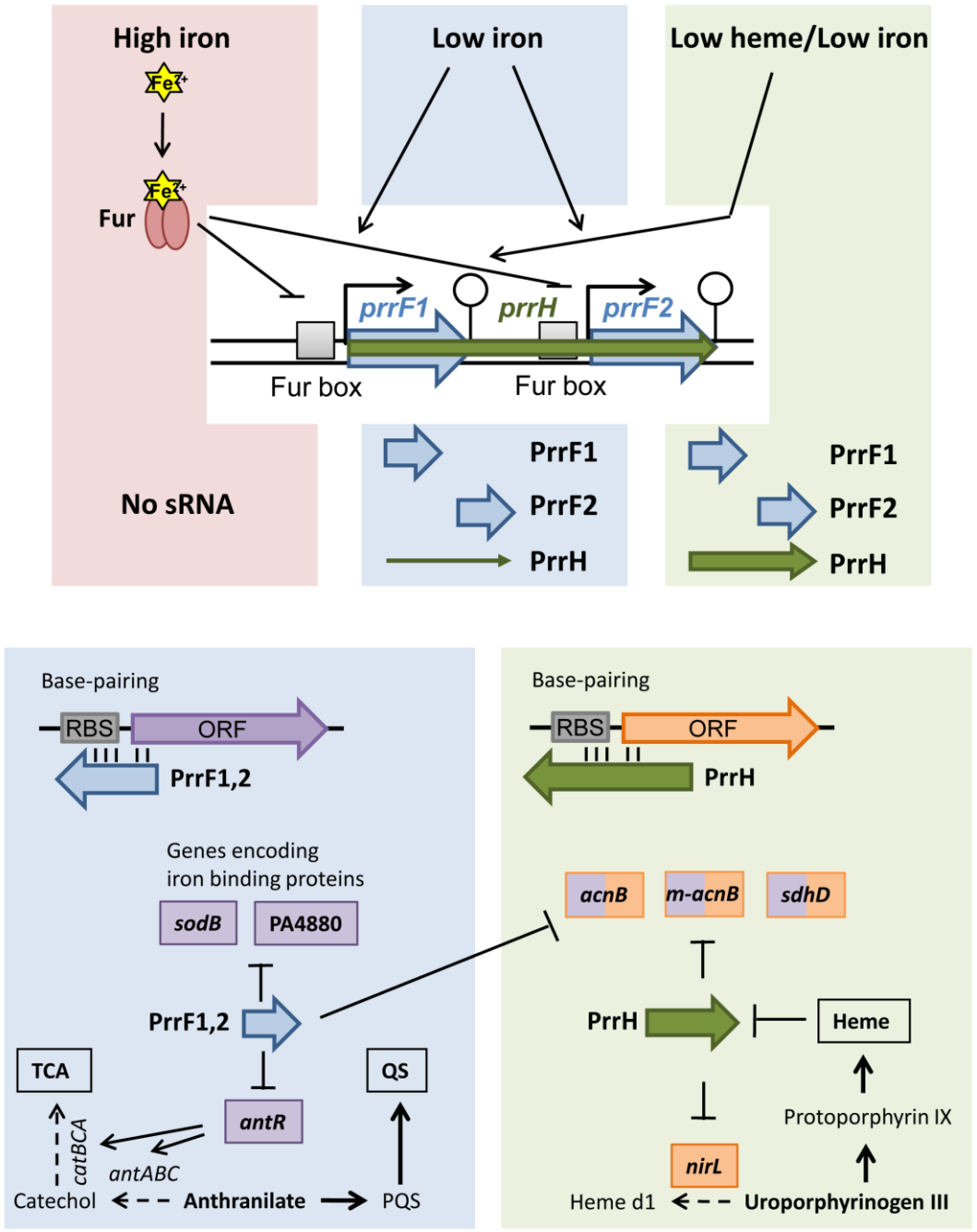


Figure 7. Model of iron- and heme regulation by the sRNAs PrrF1, PrrF2 and PrrH. Under iron-replete conditions (red box), transcription from the *prrF1/prrF2* promoter is repressed by metallo-Fur. When iron is scarce (blue box) Fur is inactive, which leads to transcription of *prrF1/prrF2*. Most likely, both sRNAs (blue arrows) repress translation initiation by base-pairing with target mRNAs, like *sodB*, PA4880, *acnB*, *m-acnB* and *sdhD*, encoding iron binding proteins, or *antR*, encoding a transcriptional activator required for anthranilate and catechol degradation. As anthranilate is a precursor for PQS synthesis, inhibition of anthranilate degradation favors PQS synthesis and quorum sensing (QS) activation. When the availability of iron and heme is limited (green box) the sRNA PrrH (dark green arrow) is transcribed from the *prrF1* promoter, most likely involving anti-termination at the *prrF1* terminator. PrrH is thought to act similar to PrrF1/PrrF2, i.e. by repressing common PrrF and PrrH targets (e.g. *acnB*, *m-acnB*, *sdhD*). In addition, PrrH apparently represses *nirL*, encoding a heme d1 biosynthesis protein. For repression of *nirL*, PrrH is presumably using the RNA sequence in between PrrF1 and PrrF2. Repression of heme d1 synthesis could favor heme production (see text). ↓: positive control; ⊥: negative control; dashed lines: indirect control.

Apart from iron metabolism, Oglesby *et al.* (2008) reported that PrrF represses *P. aeruginosa* genes involved in anthranilate and catechol degradation. Under iron-limiting conditions PrrF most likely base-pairs with the *antA* and *antR* mRNAs and prevents their translation. AntR is a transcriptional activator required for transcription of the *antABC* operon and possibly for the *catBCA* operon. In this way, the conversion of anthranilate to catechol is reduced and anthranilate is channeled into the biosynthetic pathway of the *Pseudomonas* quinolone quorum-sensing signal (PQS). In support of the proposed indirect regulation of PQS synthesis by PrrF, a *P. aeruginosa* Δ*prrF1/prrF2* mutant shows a decreased production of PQS. Hence, the PrrF sRNAs link iron metabolism to carbon metabolism and PQS signalling in *P. aeruginosa*, and thus virulence, as the latter affects the expression of several extracellular virulence factors (Heeb *et al.*, 2011).

3.5.2.2. PrrH

P. aeruginosa can take up iron by the acquisition of heme, which is an abundant source of iron in mammals. As a surplus of heme -like iron- can cause oxidative damage, heme uptake and homeostasis is highly regulated. The recently discovered *P. aeruginosa* sRNA PrrH (Figure 7) is encoded within the *prrF1/prrF2* locus, and appears to be induced by heme-regulated antitermination of *prrF1* transcription (Oglesby-Sherrouse & Vasil, 2010). The mechanism by which heme negatively affects PrrH expression is currently unknown, although the outer membrane heme receptors PhuR and HasR seem to play a role (Oglesby-Sherrouse & Vasil, 2010).

The model put forward by Oglesby-Sherrouse and Vasil (2010) entails that the PrrH and the PrrF regulons share common biological functions in oxidative stress protection, iron storage and in regulation of iron-containing enzymes. Induction of PrrH in response to iron or heme starvation leads to repression of the same genes controlled by PrrF under iron limita-

tion. However, PrrH appears to regulate also a specific subset of PrrF-independent genes, which has been suggested to be mediated by PrrF sequences located in the intergenic region between the *prrF1* and *prrF2* genes. *In-silico* target prediction and quantitative PCR suggested that *nirL* expression is repressed by PrrH under iron and heme limitation. As part of the *nirSMCFDLGHJEN* gene cluster, *nirL* encodes a protein involved in the biosynthesis of heme, a prosthetic group of the dissimilatory nitrite reductase NirS (Kawasaki *et al.*, 1997). Therefore, PrrH induction upon heme limitation could favour *de novo* synthesis of heme from uroporphyrinogen III by repressing the branching pathway of heme d1 synthesis (Oglesby-Sherrouse & Vasil, 2010). Further studies are necessary to clarify the impact of PrrH on the entire heme d1 biosynthesis pathway as well as on the denitrification pathway. The *in silico* target predictions for PrrF1/PrrF2 and PrrH correlated with transcriptome analyses and expression studies (Oglesby *et al.*, 2008; Oglesby-Sherrouse & Vasil, 2010). However, so far no extensive mutational analyses to verify the proposed base-pairing interactions between the sRNAs and mRNA targets have been performed.

3.5.2.3. PhrS

The *P. aeruginosa* PhrS (Figure 8) is a 213 nt long sRNA, which was predicted by biocomputation (Livny *et al.*, 2006; Gonzalez *et al.*, 2008) and isolated by an RNomics approach in combination with Hfq-mediated co-immunopreciptation (Sonnleitner *et al*., 2008). Expression of *phrS* occurs in stationary phase under oxygen-limiting conditions (Sonnleitner *et al.*, 2008) and requires the oxygen-responsive regulator ANR (Sonnleitner *et al*., 2011). The steady state levels of PhrS are reduced in a *P. aeruginosa hfq* mutant, although Hfq is not affecting the stability of PhrS (Sonnleitner *et al.*, 2006). Subsequent studies showed that Hfq is required for ANR expression, whereby the underlying mechanism remains unclear (Sonnleitner *et al.*, 2011). Proteome analyses upon *phrS* over-expression revealed an up-regulation of the *P. ae-*

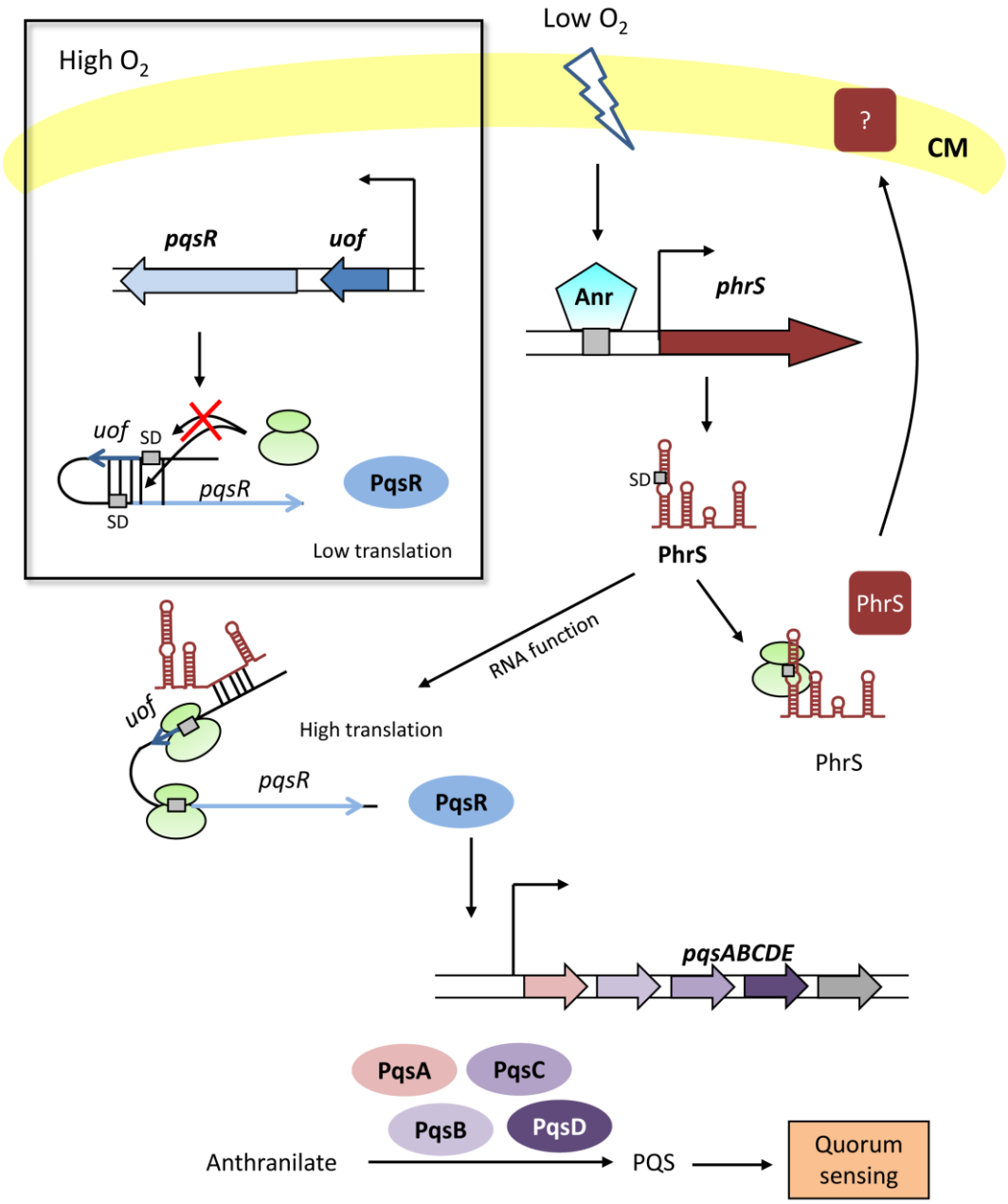


Figure 8. Quorum sensing regulation by PhrS. At high oxygen levels, translation of *pqsR*, a key regulator of quorum sensing, is impeded by a secondary structure sequestering the Shine and Dalgarno sequence, resulting in low translation of *pqsR*. When oxygen becomes limited, the oxygen-responsive transcriptional regulator Anr is activated and directs transcription of the sRNA PhrS. PhrS base-pairs with the leader sequence of the upstream open reading frame (*uof*), which is co-transcribed with *pqsR*. The resulting changes in mRNA secondary structure permit translation of *uof* and - as *uof* and *pqsR* are positively translationally coupled - that of *pqsR*. The PqsR protein directs expression of the PQS biosynthesis operon. The encoded enzymes convert anthranilate into the quorum sensing signaling molecule PQS. Besides acting as a base-pairing RNA, PhrS encodes a short cytoplasmic membrane (CM) protein with unknown function.

ruginosa genes encoding the GroEL chaperone, the OprD outer membrane protein, and a putative periplasmic binding protein (Sonnleitner *et al.*, 2008). More intriguingly, an increase in the synthesis of both, the Pseudomonas quinolone signal (PQS) and pyocyanin (PYO), was

dependent on the presence of PhrS (Sonnleitner *et al.*, 2011). Given that all PhrS-dependent genes involved in PYO and PQS synthesis are under positive control of the transcriptional regulator PqsR (Déziel *et al.*, 2005), the effect of PhrS on *pqsR* mRNA was investigated. Although the ribosome binding site of *pqsR* was at least partially sequestered by secondary structure (Sonnleitner *et al.*, 2011), no evidence was found for PhrS anti-anti-sense pairing in the translation initiation region of the *pqsR* gene. Instead, a short upstream open reading frame (*uof*) was identified as the primary target of the sRNA, and structural probing experiments showed that the ribosome binding site of *uof* - like that of *pqsR* - is partially masked by secondary structure. Moreover, mutational analyses of PhrS and the *uof-pqsR* mRNA supported the inhibitory structures that repress *uof* and *pqsR* translation. A highly conserved region within PhrS was implied in base-pairing with sequences upstream of the *uof* ribosome binding site (Sonnleitner *et al.*, 2011). As translation of *pqsR* was shown to be positively coupled to that of *uof*, PhrS-mediated up-regulation of *uof* translation stimulates consequently translation of *pqsR*.

3.6. Multifunctional sRNAs: the advantages of functional plasticity

In addition to its function as a regulatory RNA, PhrS encodes a highly conserved 37 amino-acid polypeptide (Sonnleitner *et al.*, 2008). Computer algorithms predicted one transmembrane segment for the PhrS-peptide and sub-cellular fractionation revealed that the peptide is residing in the cytoplasmic membrane. However, the PhrS-peptide appears not to affect release of PQS, and it is therefore questionable whether it acts within the same regulatory circuit as the base-pairing function of PhrS (E. Sonnleitner, unpublished). Moreover, it is also unclear whether the *uof*-encoded 40 amino-acid peptide has any cellular function or whether its synthesis merely ensures stimulation of *pqsR* translation during hypoxia.

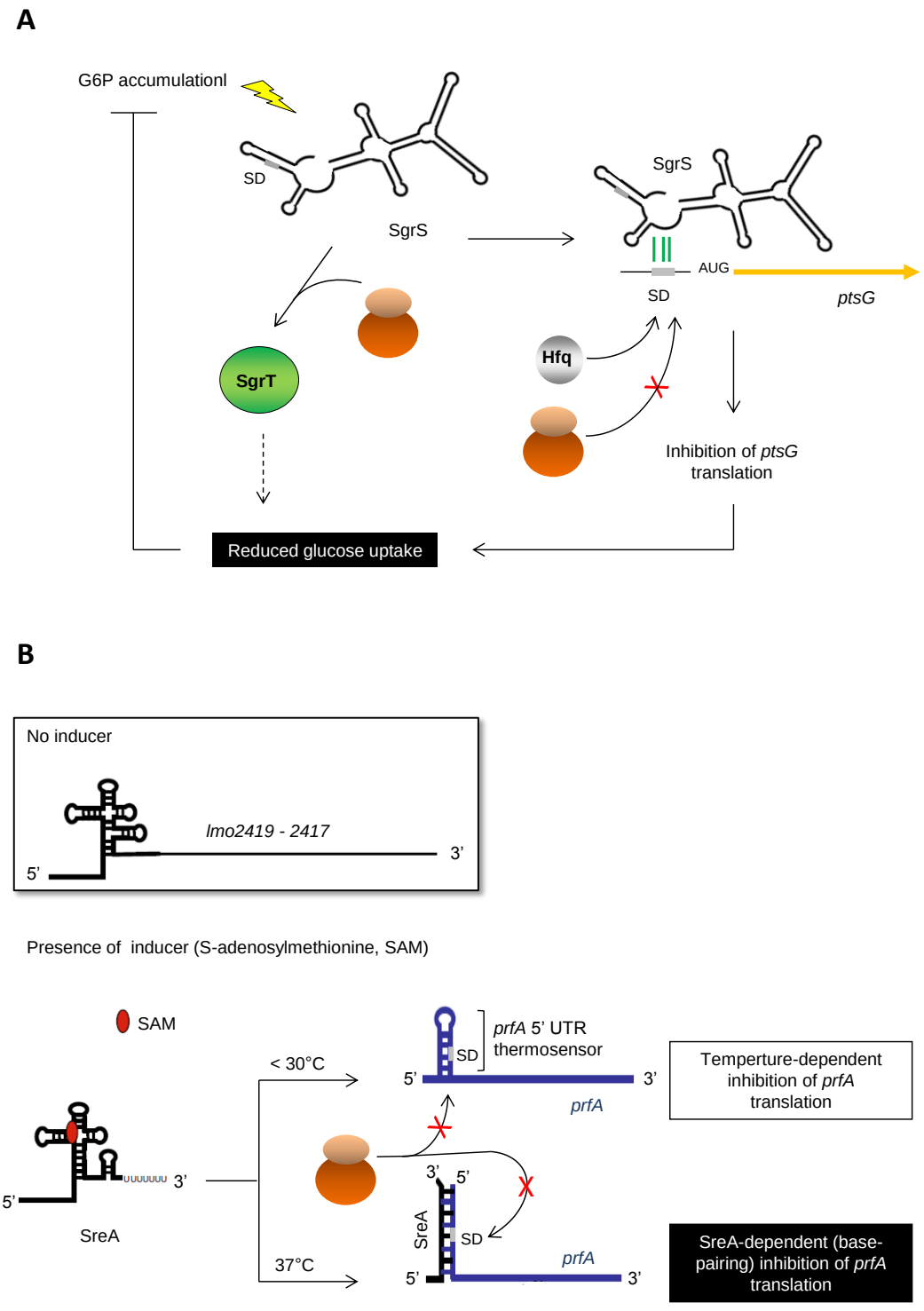


Figure 9. Examples of bifunctional sRNAs. (A) In *E. coli*, the phosphosugar stress-induced sRNA SgrS inhibits further glucose uptake by inhibiting the translation of the major glucose transport gene *ptsG* through a base-pairing mechanism. The ORF within SgrS is simultaneously translated into the protein SgrT, which indirectly reduces further glucose uptake. (B) In *Listeria monocytogenes*, the *sre* transcripts (i.e. SreA) acts as both, as a *cis* and as a *trans* regulatory RNAs. As a riboswitch, SreA controls expression of the downstream *lmo2419-2417* genes. Upon starvation conditions, the presence of the ligand SAM terminates transcription at the *sreA* terminator. These SreA independent transcripts inhibit translation of the virulence gene *prfA* mRNA through base-pairing with the thermosensor located in the 5' end of *prfA* mRNA at 37°C.

In *E. coli*, the 227 nucleotides long sRNA SgrS is expressed during glucose-induced accumulation of phosphosugars, such as glucose-6-phosphate (Figure 9A). Under these stress conditions, SgrS binds to and reduces translation of its target mRNA *ptsG* (Vanderpool & Gottesman, 2004), which encodes the major glucose transport of the phosphoenolpyruvate phosphotransferase system (PTS, Kimata *et al.*, 2001; Morita *et al.*, 2003). The base-pairing between SgrS and *ptsG* is assisted by the RNA chaperone Hfq (Zhang *et al.*, 2003). SgrS encodes a 43 amino acid peptide which is translated under glucose-phosphate stress conditions. The product of this ORF, named SgrT, inhibits glucose uptake through a PtsG-independent regulation (Wadler & Vanderpool, 2007; Figure 9A).

Another example in *E. coli* is illustrated by the GraL small transcripts array generated by differential termination at the *greA* leader RNA (Potrykus *et al.*, 2010). These evolutionary conserved 40 to 60 nucleotides long small RNAs were shown to impact on *E. coli* fitness and transcriptome.

The master regulator PrfA, which is required for virulence genes expression in *Listeria monocytogenes*, is post-transcriptionally regulated by a thermosensor, which is located in the 5' UTR of the *prfA* transcript. While allowing translation of the mRNA at 37°C, the secondary structure masks the ribosome binding site at temperatures of 30°C or below (Johansson *et al.*, 2002; Figure 9B). The regulation of *prfA* translation is further dependent on nutrient availability. Loh and colleagues (2009) detected the presence of a small transcript termed the *sre* riboswitch (S-adenosylmethionine riboswitch element), generated by ligand-induced termination of the longer *lmo2419-2417* transcript. These *sre* transcripts (Figure 9B) reduce translation of *prfA* through base-pairing with the ribosome binding site of *prfA* under permissive temperatures (37°C). Consequently, the *sre* riboswitches control the synthesis of its targets *lmo2419-2417* and *prfA* in *cis* and *trans*, respectively (Figure 9B).

3.7. Aims of the Work

The aim of this PhD-thesis was to elucidate the function of the *P. aeruginosa* RNA NalA. NalA was discovered during a bioinformatic screen for novel small RNAs. Reporter gene assays and Northern-blot analyses were used to monitor the NalA levels during different growth conditions. The *nalA* promoter was studied by mutational analysis.

As *nalA* is located proximal of the *P. aeruginosa* nitrate/nitrite assimilation operon, the role of NalA as a putative *cis*-regulatory RNA element was investigated. In addition, the question was addressed whether NalA can as well function as a *trans*-regulatory RNA in PAO1. The transcriptomes of a PAO1Δ*nalA* strain and a *nalA* over-expressing PAO1 strain were compared using DNA microarrays. Then, follow-up experiments were performed to verify regulation of putative target genes by NalA.

3.8. References

Adler, S. P., Purich, D. & Stadtman, E. R. (1975). Cascade control of *Escherichia coli* glutamine synthetase. Properties of the P_{II} regulatory protein and the uridylyltransferase-uridylyl-removing enzyme. *J Biol Chem* **250(16)**, 6264-6272.

Al-Attar, S., Westra, E. R., van der Oost, J. & Brouns, S. J. (2011). Clustered regularly interspaced short palindromic repeats (CRISPRs): the hallmark of an ingenious antiviral defense mechanism in prokaryotes. *Biol Chem* **392**, 277-289.

Alonso, A., Campanario, E. & Martinez, J. L. (1999). Emergence of multidrug- resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. *Microbiology (UK)* **145**, 2857-2862.

Arai, H., Igarashi, Y. & Kodama, T. (1994). Structure and ANR-dependent transcription of the *nir* genes for denitrification from *Pseudomonas aeruginosa*. *Biosci Biotechnol Biochem* **58(7)**, 1286-1291.

Arai, H., Igarashi, Y. & Kodama, T. (1995). Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett* **371(1)**, 73-76.

Austin, S. & Dixon, R. (1992). The prokaryotic enhancer binding protein NTRC has an ATPase activity which is phosphorylation and DNA dependent. *EMBO J* **11(6)**, 2219-2228.

Babitzke, P. & Romeo, T. (2007). CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr Opin Microbiol* **10**, 156-163.

Barth, A. L. & Pitt, T. L. (1996). The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*. *J Med Microbiol* **45(2)**, 110-119.

Beckmann, C., Brittnacher, M., Ernst, R., Mayer-Hamblett, N., Miller, S. I. & Burns, J .L. (2005). Use of phage display to identify potential *Pseudomonas aeruginosa* gene products relevant to early cystic fibrosis airway infections. *Infect Immun* **73**, 444-452.

Bedzyk, L., Wang, T. & Ye, R. W. (1999). The periplasmic nitrate reductase in *Pseudomonas* sp. strain G-179 catalyzes the first step of denitrification. *J Bacteriol* **181(9)**, 2802-2806.

Benkert, B., Quack, N., Schreiber, K., Jaensch, L., Jahn, D. & Schobert, M. (2008). Nitrate-responsive NarX-NarL represses arginine-mediated induction of the *Pseudomonas aeruginosa* arginine fermentation *arcDABC* operon. *Microbiology* **154**, 3053-3060.

Bentley, S. D. & Parkhill, J. (2004). Comparative genomic structure of prokaryotes. *Annu Rev Genet* **38**, 771-792.

Berks, B. C., Ferguson, S. J., Moir, J. W. B. & Richardson, D. J. (1995). Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim Biophys Acta* **1232**, 97-173.

Blasco, R., Castillo, F. & Martinez-Luque, M. (1997). The assimilatory nitrate reductase from the phototrophic bacterium, *Rhodobacter capsulatus* E1F1, is a flavoprotein. *FEBS Lett* **414**, 45-49.

Bodey, G. P., Bolivar, R., Fainstein, V. & Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis* **5(2)**, 279-313.

Bordi, C., Lamy, M. C., Ventre, I., Termine, E., Hachani, A., Fillet, S., Roche, B., Bleves, S., Méjean, V. & other authors. (2010). Regulatory RNAs and the HptB/RetS signalling pathways fine-tune *Pseudomonas aeruginosa* pathogenesis. *Mol Microbiol* **76**, 1427-1443.

Branda, S. S., Vik, S., Friedman, L. & Kolter, R. (2005). Biofilms: the matrix revisited. *Trends Microbiol* **13(1)**, 20-26.

Breidenstein, E. B. M., de la Fuente-Núñez, C. & Hancock, R. E. W. (2011). *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends in Microbiology* **19(8)**.

Brencic, A. & Lory, S. (2009). Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* **72**, 612-632.

Brencic, A., McFarland, K. A., McManus, H. R., Castang, S., Mogno, I., Dove, S. L. & Lory, S. (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**, 434-445.

Brondijk, T. H., Fiegen, D., Richardson, D. J. & Cole, J. A. (2002). Roles of NapF, NapG and NapH, subunits of the *Escherichia coli* periplasmic nitrate reductase, in ubiquinol oxidation. *Mol Microbiol* **44(1)**, 245-255.

Brown, M. S., Segal, A. & Stadtman, E. R. (1971). Modulation of glutamine synthetase adenylylation and deadenylylation is mediated by metabolic transformation of the P_{II} -regulatory protein. *Proc Natl Acad Sci USA* **68(12)**, 2949-2953.

Burrowes, E., Baysse, C., Adams, C. & O'Gara, F. (2006). Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. *Microbiology* **152**, 405-418.

Cabello, P., Roldán, M. D. & Moreno-Vivián, C. (2004). Nitrate reduction and the nitrogen cycle in archaea. *Microbiology* **150**, 3527-3546.

Cady, K. C. & O'Toole, G. A. (2011). Non-identity-mediated CRISPR-bacteriophage interaction mediated via the Csy and Cas3 proteins. *J Bacteriol* **193(14)**, 3433-3445.

Cady, K. C., White, A. S., Hammond, J. H., Abendroth, M. D., Karthikeyan, R. S., Lalitha, P., Zegans, M. E. & O'Toole, G. A. (2011). Prevalence, conservation and functional analysis of *Yersinia* and *Escherichia* CRISPR regions in clinical *Pseudomonas aeruginosa* isolates. *Microbiology* **157**, 430-437.

Campbell, W. H. & Kinghorn, J. R. (1990). Functional domains of assimilatory nitrate reductases and nitrite reductases. *Trends Biochem Sci* **15**, 315-319.

Carlson, C. A. & Ingraham, J. L. (1983). Comparison of denitrification by *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, and *Paracoccus denitrifi*cans. *Appl Environ Microbiol* **45**, 1247-1253.

Castiglione, N., Rinaldo, S., Giardina, G. & Cutruzzolà, F. (2009). The transcription factor DNR from *Pseudomonas aeruginosa* specifically requires nitric oxide and haem for the activation of a target promoter in *Escherichia coli*. *Microbiology* **155(Pt9)**, 2838-2844.

Chai, W. & Stewart, V. (1998). NasR, a novel RNA-binding protein, mediates nitrate responsive transcription antitermination of the *Klebsiella oxytoca* M5al *nasF* operon leader *in vitro*. *J Mol Biol* **283**, 339-351.

Colandene, J. D. & Garrett, R. H. (1996). Functional dissection and site-directed mutagenesis of the structural gene for NAD(P)H-nitrite reductase in *Neurospora crassa*. *J Biol Chem* **271(39)**, 24096-24104.

Contreras, A. & Drummond, M. (1988). The effect on the function of the transcriptional activator NtrC from *Klebsiella pneumoniae* of mutations in the DNA-recognition helix. *Nucleic Acids Res* **16(9)**, 4025-4039.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280(5361)**, 295-298.

Davies, K. J., Lloyd, D. & Boddy, L. (1989). The effect of oxygen on denitrification in *Paracoccus denitrificans* and *Pseudomonas aeruginosa*. *J Gen Microbiol* **135**, 2445-2451.

de Kievit, T. R. (2009). Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* **11(2)**, 279-88.

Déziel, E., Gopalan, S., Tampakaki, A. P., Lépine, F., Padfield, K. E., Saucier, M., Xiao, G. & Rahme, L. G. (2005). The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of N-acyl-L-homoserine lactones. *Mol Microbiol* **55**, 998-1014.

Dietrich, L. E., Teal, T. K., Price-Whelan, A. & Newman, D. K. (2008). Redox-active antibiotics control gene expression and community behavior in divergent Bacteria. *Science* **321(5893)**, 1203-1206.

Dixon, R (1984). Tandem promoters determine regulation of the *Klebsiella pneumoniae* glutamine synthetase (*glnA*) gene. *Nucleic Acids Res* **12(20)**, 7811-7830.

Donaldson, S. H. & Boucher, R. C. (2007). Sodium channels and cystic fibrosis. *Chest* **132(5)**, 1631-1636.

Doring, D. (1993). *Pseudomonas aeruginosa* as an opportunistic pathogen. New York: Plenum Press; Chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. pp. 245-273

Downey, D. & Elborn, J. S. (2000). Nitric oxide, iNOS, and inflammation in cystic fibrosis. *J Pathol* **190(2)**, 115-116.

Einsle, O. & Kroneck, P. M. (2004). Structural basis of denitrification. *Biol Chem* **385(10)**, 875-883.

Engleman, E. & Francis, S. (1978). Cascade control of *E. coli* glutamine synthetase. II. Metabolite regulation of the enzymes in the cascade. *Arch Biochem Biophys* **191**, 602-612.

Eschbach, M., Schreiber, K., Trunk, K., Buer, J., Jahn, D. & Schobert, M. (2004). Long- term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *J Bacteriol* **186**, 4596-4604.

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

Filloux, A. (2004). The underlying mechanisms of type II protein secretion. *Biochim Biophys Acta* **1694**, 163-179.

Flanagan, D. A., Gregory, L. G., Carter, J. P., Karakas-Sen, A., Richardson, D. J. & Spiro, S. (1999). Detection of genes for periplasmic nitrate reductase in nitrate respiring Bacteria and in community DNA. *FEMS Microbiol Lett* **177(2)**, 263-270.

Fleiszig, S. M., Wiener-Kronish, J. P., Miyazaki, H., Vallas, V., Mostov, K. E., Kanada, D., Sawa, T., Yen, T. S. & Frank, D. W. (1997). *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infect Immun* **65(2)**, 579-586.

Furukawa, S., Kuchma, S. L. & O'Toole, G. A. (2006). Keeping their options open: acute versus persistent infections. *J Bacteriol* **188**, 1211-1217.

Gavira, M., Roldan, M. D., Castillo, F. & Moreno-Vivián, C. (2002). Regulation of *nap* gene expression and periplasmic nitrate reductase activity in the phototrophic bacterium *Rhodobacter sphaeroides* DSM158. *J Bacteriol* **184**, 1693-1702.

Geissmann, T. A. & Touati, D. (2004). Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J* **23**, 396-405.

Giardina, G., Rinaldo, S., Castiglione, N., Caruso, M. & Cutruzzolà, F. (2009). A dramatic conformational rearrangement is necessary for the activation of DNR from *Pseudomonas aeruginosa*. Crystal structure of wild-type DNR. *Proteins* **77(1)**, 174-180.

Giardina, G., Rinaldo, S., Johnson, K. A., Di Matteo, A., Brunori, M. & Cutruzzolà, F. (2008). NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *J Mol Biol* **378**, 1002–1015.

Goldman, B. S., Lin, J. T. & Stewart, V. (1994). Identification and structure of the *nasR* gene encoding a nitrate- and nitrite-responsive positive regulator of *nasFEDCBA* (nitrate assimilation) operon expression in *Klebsiella pneumoniae* M5a1. *J Bacteriol* **176**, 5077-5085.

Gonzalez, 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.

Goodman, A. L., Merighi, M., Hyodo, M., Ventre, I., Filloux, A. & Lory, S. (2009). Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev* **23**, 249-259.

Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S. & Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* **7**, 745-754.

Görke, B. & Stülke, J. (2008). Carbon catabolite repression in Bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* **6**, 613-624.

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

Govan, J. R. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**, 539-574.

Gregory, L. G., Bond, P. L., Richardson, D. J. & Spiro, S. (2003). Characterization of a nitrate-respiring bacterial community using the nitrate reductase gene (*narG*) as a functional marker. *Microbiology* **149**, 229-237.

Gutierrez, J. C., Ramos, F., Ortner, L. & Tortolero, M. (1995). *nasST*, two genes involved in the induction of the assimilatory nitrite-nitrate reductase operon (*nasAB*) of *Azotobacter vinelandii*. *Mol Microbiol* **18(3)**, 579-591.

Hall-Stoodley, L. & Stoodley, P. (2009). Evolving concepts in biofilm infections. *Cell Microbiol* **11(7)**, 1034-1043.

Hantke, K. (2001). Iron and metal regulation in Bacteria. *Curr Opin Microbiol* **4**, 172-177.

Hayashi, N. R., Arai, H., Kodama, T. & Igarashi, Y. (1998). The *nirQ* gene, which is required for denitrification of *Pseudomonas aeruginosa*, can activate the RubisCO from *Pseudomonas hydrogenothermophila*. *Biochim Biophys Acta* **1381**, 347-350.

Heeb, S., Blumer, C. & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* **184**, 1046-1056.

Heeb, S., Fletcher, M. P., Chabra, S. R., Diggle, S. P., Williams, P. & Camara, M. (2011). Quinolones: from antibiotics to autoinducers. *FEMS Microbiol Rev* **35**, 247-274.

Heeb, S., Kuehne, S. A., Bycroft, M., Crivii, S., Allen, M. D., Haas, D., Cámara, M. & Williams, P. (2006). Functional analysis of the post-transcriptional regulator RsmA reveals a novel RNA-binding site. *J Mol Biol* **355**, 1026-1036.

Høiby, N., Ciofu, O., Johansen, H. K., Song, Z. J., Moser, C., Jensen, P. Ø., Molin, S., Givskov, M., Tolker-Nielsen, T. & other authors. (2011). The clinical impact of bacterial biofilms. *Int J Oral Sci* **3(2)**, 55-65.

Hsu, J. L., Chen, H. C., Peng, H. L. & Chang, H. Y. (2008). Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in *Pseudomonas aeruginosa* PAO1. *J Biol Chem* **283**, 9933-9944.

Hu, B. L., Shen, L. D., Xu, X. Y. & Zheng, P. (2012). Anaerobic ammonium oxidation (anammox) in different natural ecosystems. *Biochem Soc Trans* **39(6)**, 1811-1816.

Hudson, R. C. & Daniel, R. M. (1993). L-glutamate dehydrogenases: distribution, properties and mechanism. *Comp Biochem Physiol B* **106(4)**, 767-792.

Humair, B., Wackwitz, B. & Haas, D. (2010). GacA-controlled activation of promoters for small RNA genes in *Pseudomonas fluorescens*. *Appl Environ Microbiol* **76**, 1497-1506.

Jain, S., Franklin, M. J., Ertesvåg, H., Valla, S. & Ohman, D. E. (2003). The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. *Mol Microbiol* **47(4)**, 1123-1133.

Jeter, R. M., Sias, S. R. & Ingraham, J. L. (1984). Chromosomal location and function of genes affecting *Pseudomonas aeruginosa* nitrate assimilation. *J Bacteriol* **157(2)**, 673-677.

Jiang, P., Peliska, J. A. & Ninfa, A. J. (1998). The regulation of *Escherichia coli* glutamine synthetase revisited: role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state. *Biochemistry* **37(37)**, 12802-12810.

Jing, X., Jaw, J., Robinson, H. H. & Schubot, F. D. (2010). Crystal structure and oligomeric state of the RetS signaling kinase sensory domain. *Proteins* **78**, 1631-1640.

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M. & Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110**, 551-561.

Kaberdin, V. R. & Bläsi, U. (2006). Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol Rev* **30**, 967-979.

Kamberov, E. S., Atkinson, M. R. & Ninfa, A. J. (1995). The *Escherichia coli* P_{II} signal transduction protein is activated upon binding 2-ketoglutarate and ATP. *J Biol Chem* **270**, 17797-17807.

Kawasaki, S., Arai, H., Kodama, T. & Igarashi, Y. (1997). Gene cluster for dissimilatory nitrite reductase (*nir*) from *Pseudomonas aeruginosa*: sequencing and identification of a locus for heme d1 biosynthesis. *J Bacteriol* **179**, 235-242.

Kay, E., Dubuis, C. & Haas, D. (2005). Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. *Proc Natl Acad Sci USA* **102**, 17136-17141.

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.

Kazantsev, A. V. & Pace, N. R. (2006). Bacterial RNase P: a new view of an ancient enzyme. *Nat Rev Microbiol* **4**, 729-740.

Keener, J. & Kustu, S. (1988). Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric Bacteria: roles of the conserved amino-terminal domain of NTRC. *Proc Natl Acad Sci USA* **85**, 4976-4980.

Kim, J. & Rees, D. C. (1994). Nitrogenase and biological nitrogen fixation. *Biochemistry* **33(2)**, 389-397.

Kimata, K., Tanaka, Y., Inada, T. & Aiba, H. (2001). Expression of the glucose transporter gene, *ptsG*, is regulated at the mRNA degradation step in response to glycolytic flux in *Escherichia coli*. *EMBO J* **20**, 3587-3595.

King, J. D., Kocincova, D., Westman, E. L. & Lam, J. S. (2009). Review: lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. *Innate Immun* **15**, 261-312.

Klotz, M. G. & Stein, L. Y. (2008). Nitrifier genomics and evolution of the nitrogen cycle. *FEMS Microbiol Lett* **278**, 146-156.

Kobayashi, H., Kobayashi, O. & Kawai, S. (2009). Pathogenesis and clinical manifestations of chronic colonization by *Pseudomonas aeruginosa* and its biofilms in the airway tract. *J Infect Chemother* **15(3)**, 125-142.

Kustu, S., Hirschman, J., Burton, D., Jelesko, J. & Meeks, J. C. (1984). Covalent modification of Bacterial glutamine synthetase: physiological significance. *Mol Gen Genet* **197(2)**, 309-317.

Lapouge, K., Schubert, M., Allain, F. H. & Haas, D. (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol Microbiol* **67**, 241-253.

Lapouge, K., Sineva, E., Lindell, M., Starke, K., Baker, C. S., Babitzke, P. & Haas, D. (2007). Mechanism of *hcnA* mRNA recognition in the Gac/Rsm signal transduction pathway of *Pseudomonas fluorescens*. *Mol Microbiol* **66**, 341-356.

Laskowski, M. A. & Kazmierczak, B. I. (2006). Mutational analysis of RetS, an unusual sensor kinase-response regulator hybrid required for *Pseudomonas aeruginosa* virulence. *Infect Immun* **74**, 4462-4473.

Laskowski, M. A., Osborn, E. & Kazmierczak, B. I. (2004). A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*. *Mol Microbiol* **54**, 1090-1103.

Li, W. & Lu, C.-D. (2007). Regulation of carbon and nitrogen utilization by CbrAB and NtrBC two-component systems in *Pseudomonas aeruginosa. J Bacteriol* **189**, 5413-5420.

Li, Z., Kosorok, M. R., Farrell, P. M., Laxova, A., West, S. E., Green, C. G., Collins, J., Rock, M. J. & Splaingard, M. L. (2005). Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. *JAMA* **293(5)**, 581-588.

Lin, J. T. & Stewart, V. (1996). Nitrate and nitrite-mediated transcription antitermination control of *nasF* (nitrate assimilation) operon expression in *Klebsiella pneumoniae* M5a1. *J Mol Biol* **256**, 423-435.

Lin, J. T. & Stewart, V. (1998). Nitrate assimilation by Bacteria. *Adv Microb Physiol* **39**, 1-30, 379.

Linares, J. F., Moreno, R., Fajardo, A., Martínez, L., Escalante, R., Rojo, F. & Martínez, J. L. (2010). The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. *Environ Microbiol* **12**, 3196-3212.

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.

Loh, E., Dussurget, O., Gripenland, J., Vaitkevicius, K., Tiensuu, T., Mandin, P., Repoila, F., Buchrieser, C., Cossart, P. & other authors (2009). A *trans*-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*. *Cell* **139(4)**, 770-779.

Luque, I., Flores, E. & Herrero, A. (1993). Nitrite reductase gene from *Synechococcus* sp. PCC 7942: homology between cyanobacterial and higher-plant nitrite reductases. *Plant Mol Biol* **21**, 1201-1205.

Luthi, E., Baur, H., Gamper, M., Brunner, F., Villeval, D., Mercenier, A. & Haas, D. (1990). The *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa* contains an additional gene, *arcD*, encoding a membrane protein. *Gene* **87**, 37-43.

MacFarlane, S. A. & Merrick, M. (1985). The nucleotide sequence of the nitrogen regulation gene *ntrB* and the *glnA-ntrBC* intergenic region of *Klebsiella pneumoniae*. *Nucleic Acids Res* **13(21)**, 7591-7606.

Magalon, A., Asso, M., Guigliarelli, B., Rothery, R. A., Bertrand, P., Giordano, G. & Blasco, F. (1998). Molybdenum cofactor properties and [Fe-S] cluster coordination in *Escherichia coli* nitrate reductase A: investigation by site-directed mutagenesis of the conserved His-50 residue in the NarG subunit. *Biochemistry* **37**, 7363-7370.

Mah, T. F. & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9(1)**, 34-39.

Mahenthiralingam, E., Campbell, M. E. & Speert, D. P. (1994). Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* **62(2)**, 596-605.

Mangum, J. H., Magni, G. & Stadtman, E. R. (1973). Regulation of glutamine synthetase adenylylation and deadenylylation by the enzymatic uridylylation and deuridylylation of the P_{II} regulatory protein. *Arch Biochem Biophys* **158(2)**, 514-525.

Manos, J., Arthur, J., Rose, B., Bell, S., Tingpej, P., Hu, H., Webb, J., Kjelleberg, S., Gorrell, M. D. & other authors. (2009). Gene expression characteristics of a cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* during biofilm and planktonic growth. *FEMS Microbiol Lett* **292**, 107-114.

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

Matz, C. & Kjelleberg, S. (2005). Off the hook--how bacteria survive protozoan grazing. *Trends Microbiol* **13(7)**, 302-307.

Massé, E., Vanderpool, C. K. & Gottesman, S. (2005). Effect of RyhB small RNA on global iron use in *Escherichia coli*. *J Bacteriol* **187**, 6962-6971.

Mettke, I., Fiedler, U. & Weiss, V. (1995). Mechanism of activation of a response regulator: interaction of NtrC-P dimers induces ATPase activity. *J Bacteriol* **177(17)**, 5056-5061.

Mikami, B. & Ida, S. (1984). Purification and properties of ferredoxin—nitrate reductase from the cyanobacterium *Plectonema boryanum*. *Biochim Biophys Acta* **791**, 294-304.

Mojica, F. J., Díez-Villaseñor, C., García-Martínez, J. & Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* **60**, 174-182.

Moore, N. M. & Flaws, M. L. (2011). Antimicrobial resistance mechanisms in *Pseudomonas aeruginosa*. *Clin Lab Sci* **24(1)**, 47-51.

Moreno, R. & Rojo, F. (2008). The target for the *Pseudomonas putida* Crc global regulator in the benzoate degradation pathway is the BenR transcriptional regulator. *J Bacteriol* **190**, 1539-1545.

Moreno, R., Fonseca, P. & Rojo, F. (2010). The Crc global regulator inhibits the *Pseudomonas putida* pWW0 toluene/xylene assimilation pathway by repressing translation of regulatory and structural genes. *J Biol Chem* **285**, 24412-24419.

Moreno, R., Marzi, S., Romby, P. & Rojo, F. (2009). The Crc global regulator binds to an unpaired A-rich motif at the *Pseudomonas putida alkS* mRNA coding sequence and inhibits translation initiation. *Nucl Acids Res* **37**, 7678-7690.

Moreno, R., Ruiz-Manzano, A., Yuste, L. & Rojo, F. (2007). The *Pseudomonas putida* Crc global regulator is an RNA binding protein that inhibits translation of the AlkS transcriptional regulator. *Mol Microbiol* **64**, 665-675.

Moreno, S., Patriarca, E. J., Chiurazzi, M., Meza, R., Defez, R., Lamberti, A., Riccio, A., Iaccarino, M. & Espin G. (1992). Phenotype of a *Rhizobium leguminosarum ntrC* mutant. *Res Microbiol* **143**, 161-171.

Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R. & Castillo, F. (1999). Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. *J Bacteriol* **181(21)**, 6573-6584.

Morita, T., El-Kazzaz, W., Tanaka, Y., Inada, T. & Aiba, H. (2003). Accumulation of glucose 6-phosphate or fructose 6-phosphate is responsible for destabilization of glucose transporter mRNA in *Escherichia coli*. *J Biol Chem* **278**, 15608-15614.

Nakae, T. (1995). Role of membrane permeability in determining antibiotic resistance in *Pseudomonas aeruginosa*. *Microbiol Immunol* **39(4)**, 221-229.

Newton, W. E. (2007). Physiology, biochemistry and molecular biology of nitrogen fixation. In Biology of the nitrogen cycle (Bothe, H., Ferguson, S. J. and Newton, W. E., eds), pp. 109-129, Elsevier, Amsterdam.

Nicas, T. I. & Iglewski, B. H. (1985). The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. *Can J Microbiol* **31(4)**, 387-392.

Nilavongse, A., Brondijk, T. H., Overton, T. W., Richardson, D. J., Leach, E. R. & Cole, J. A. (2006). The NapF protein of the *Escherichia coli* periplasmic nitrate reductase system: demonstration of a cytoplasmic location and interaction with the catalytic subunit, NapA. *Microbiology* **152(Pt 11)**, 3227-3237.

Ninfa, A. J. & Bennett, R. L. (1991). Identification of the site of autophosphorylation of the bacterial protein kinase/phosphatase NRII. *J Biol Chem* **266(11)**, 6888-6893.

Ninfa, E. G., Atkinson, M. R., Kamberov, E. S. & Ninfa, A. J. (1993). Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NRII or NtrB): trans-phosphorylation between subunits. *J Bacteriol* **175(21)**, 7024-7032.

Nishijyo, T., Haas, D. & Itoh, Y. (2001). The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. *Mol Microbiol* **40**, 917-931.

Noriega, C., Hassett, D. J. & Rowe, J. J. (2005). The *mobA* gene is required for assimilatory and respiratory nitrate reduction but not xanthine dehydrogenase activity in *Pseudomonas aeruginosa*. *Curr Microbiol* **51(6)**, 419-424.

O'Toole, G. A., Gibbs, K. A., Hager, P. W., Phibbs, Jr. P. V. & Kolter, R. (2000). The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 425-431.

Ochman, H. & Davalos, L. M. (2006). The nature and dynamics of bacterial genomes. *Science* **311(5768)**, 1730-1733.

Ogawa, K., Akagawa, E., Yamane, K., Sun, Z. W., LaCelle, M., Zuber, P. & Nakano, M. M. (1995). The *nasB* operon and *nasA* gene are required for nitrate/nitrite assimilation in *Bacillus subtilis*. *J Bacteriol* **177(5)**, 1409-1413.

Oglesby, A. G., Farrow, J. M. 3rd, Lee, J. H., Tomaras, A. P., Greenberg, E. P., Pesci, E. C. & Vasil, M. L. (2008). The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. *J Biol Chem* **283**, 15558-15567.

Oglesby-Sherrouse, A. G. & Vasil, M. L. (2010). Characterization of a heme-regulated non-coding RNA encoded by the *prrF* locus of *Pseudomonas aeruginosa*. *PLoS One* **5**, e9930.

Oliver, A., Canton, R., Campo, P., Baquero, F. & Blazquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288**, 1251-1253.

Olmo-Mira, M. F., Gavira, M., Richardson, D. J., Castillo, F., Moreno-Vivián, C. & Roldán, M. D. (2004). NapF is a cytoplasmic iron-sulfur protein required for Fe-S cluster assembly in the periplasmic nitrate reductase. *J Biol Chem* **279(48)**, 49727-49735.

Palmer, K. L., Mashburn, L. M., Singh, P. K. & Whiteley, M. (2005). Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* **187**, 5267-5277.

Papenfort, K. & Vogel, J. (2009). Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level. *Res Microbiol* **160**, 278-287.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* **91(1)**, 197-201.

Pearson, J. P., Passador, L., Iglewski, B. H. & Greenberg, E. P. (1995). A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92(5)**, 1490-1494.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Cámara, 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.

Petrova, O. E. & Sauer, K. (2010). The novel two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA *rsmZ* through CafA. *J Bacteriol* **192**, 5275-5288.

Philippot, L. & Højberg, O. (1999). Dissimilatory nitrate reductases in Bacteria. *Biochim Biophys Acta* **1446(1-2)**, 1-23.

Platt, M. D., Schurr, M. J., Sauer, K., Vazquez, G., Kukavica-Ibrulj, I., Potvin, E., Levesque, R. C., Fedynak, A., Brinkman, F. S. & other authors (2008). Proteomic, microarray, and signature-tagged mutagenesis analyses of anaerobic *Pseudomonas aeruginosa* at pH 6.5, likely representing chronic, late-stage cystic fibrosis airway conditions. *J Bacteriol* **190**, 2739-2758.

Potrykus, K., Murphy, H., Chen, X., Epstein, J. A. & Cashel, M. (2010). Imprecise transcription termination within *Escherichia coli greA* leader gives rise to an array of short transcripts, GraL. *Nucleic Acids Res* **38(5)**, 1636-1651.

Potter, L., Angove, H., Richardson, D. & Cole, J. (2001). Nitrate reduction in the periplasm of Gram-negative Bacteria. *Adv Microb Physiol* **45**, 51-112.

Potter, L., Angove, H., Richardson, D. & Cole, J. (2001). Nitrate reduction in the periplasm of gram-negative bacteria. *Adv Microb Physiol* **45**, 51-112.

Pullan, S. T., Monk, C. E., Lee, L. & Poole, R. K. (2008). Microbial responses to nitric oxide and nitrosative stress: growth,"omic" and physiological methods. *Methods Enzymol* **437**, 499-519.

Ratjen, F. & Döring, G. (2003). Cystic fibrosis. *Lancet* **361(9358)**, 681-689.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. & Haas, D. (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)**, 309-319.

Reitzer, L. J. & Magasanik, B. (1983). Isolation of the nitrogen assimilation regulator NR(I), the product of the *glnG* gene of *Escherichia coli*. *Proc Natl Acad Sci USA* **80(18)**, 5554-5558.

Reyes, F., Roldán, M. D., Klipp, W., Castillo, F. & Moreno-Vivián, C. (1996). Isolation of periplasmic nitrate reductase genes from *Rhodobacter sphaeroides* DSM 158: structural and functional differences among prokaryotic nitrate reductases. *Mol Microbiol* **19**, 1307-1318.

Rhee, S. G. (1984). 5'-Nucleotidyl-O-tyrosine bond in glutamine synthetase. *Methods Enzymol* **107**, 183-200.

Richardson, D. J. & Watmough, N. J. (1999) Inorganic nitrogen metabolism in Bacteria. *Curr Opin Chem Biol* **3**, 207-219.

Rodrigue, A., Quentin, Y., Lazdunski, A., Méjean, V. & Foglino, M. (2000). Two-component systems in *Pseudomonas aeruginosa*: why so many? *Trends Microbiol* **8(11)**, 498-504.

Rodríguez-Rojas, A., Oliver, A. & Blázquez, J. (2011). Intrinsic and environmental mutagenesis drive diversification and persistence of *Pseudomonas aeruginosa* in chronic lung infections. *J Infect Dis* **205(1)**, 121-127.

Rojo, F. (2010). Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and the interaction with the environment. *FEMS Microbiol Rev* **34**, 658-784.

Rowe, J. J., Ubbink-Kok, T., Molenaar, D., Konings, W. N. & Driessen, A. J. (1994). NarK is a nitrite-extrusion system involved in anaerobic nitrate respiration by *Escherichia coli*. *Mol Microbiol* **12(4)**, 579-586.

Rubio, L. M., Flores, E. & Herrero, A. (1999). Molybdopterin guanine dinucleotide cofactor in *Synechococcus* sp. nitrate reductase: identification of *mobA* and isolation of a putative *moeB* gene. *FEBS Lett* **462(3)**, 358-362.

Rubio, L. M., Flores, E. & Herrero, A. (2002). Purification, cofactor analysis, and site-directed mutagenesis of *Synechococcus* ferredoxin-nitrate reductase. *Photosynth Res* **72**, 13-26.

Ruiz-Manzano, A., Yuste, L. & Rojo, F. (2005). Levels and activity of the *Pseudomonas putida* global regulatory protein Crc vary according to growth conditions. *J Bacteriol* **187**, 3678-3686.

Sabra, W., Lunsdorf, H. & Zeng, A. P. (2003). Alterations in the formation of lipopolysaccharide and membrane vesicles on the surface of *Pseudomonas aeruginosa* PAO1 under oxygen stress conditions. *Microbiology* **149**, 2789-2795.

Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M. & Jahn, D. (2007). The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol* **189**, 4310-4314.

Schubert, M., Lapouge, K., Duss, O., Oberstrass, F. C., Jelesarov, I., Haas, D. & Allain, F. H. (2007). Molecular basis of messenger RNA recognition by the specific bacterial repressing clamp RsmA/CsrA. *Nat Struct Mol Biol* **14**, 807-813.

Schuster, M. & Greenberg, E. P. (2006). A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* **296(2-3)**, 73-81.

Setubal, J. C., dos Santos, P., Goldman, B. S., Ertesvåg, H., Espin, G., Rubio, L. M., Valla, S., Almeida, N. F., Balasubramanian, D. & other authors. (2009). Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. *J Bacteriol* **191(14)**, 4534-4545.

Sias, S. R. & Ingraham, J. L. (1979). Isolation and analysis of mutants of *Pseudomonas aeruginosa* unable to assimilate nitrate. *Arch Microbiol* **122(3)**, 263-270.

Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P. & other authors. (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* **103(22)**, 8487-8492.

Smyth, P. F. & Clarke, P. H. (1975). Catabolite repression of *Pseudomonas aeruginosa* amidase: the effect of carbon source on amidase synthesis. *J Gen Microbiol* **90**, 81-90.

Son, H. S. & Rhee, S. G. (1987). Cascade control of *Escherichia coli* glutamine synthetase. Purification and properties of P_{II} protein and nucleotide sequence of its structural gene. *J Biol Chem* **262(18)**, 8690-8695.

Sonnleitner, E., Abdou, L. & Haas, D. (2009). Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **106**, 21866-21871.

Sonnleitner, E., Gonzalez, N., Sorger-Domenigg, T., Heeb, S., Richter, A. S., Backofen, R., Williams, P., Hüttenhofer, A. & Haas, D. (2011). The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. *Mol Microbiol* **80**, 868-885.

Sonnleitner, E., Hagens, S., Rosenau, F., Wilhelm, S., Habel, A., Jäger, 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.

Sonnleitner, E., Sorger-Domenigg, T., Madej, M. J., Findeiss, S., Hackermüller, J., Hüttenhofer, A., Stadler, P. F., Bläsi, U. & Moll, I. (2008). Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. *Microbiology* **154**, 3175-3187.

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.

Spiro, S. (2007). Regulators of bacteria responses to nitric oxide. *FEMS Microbiol Rev* **31**, 193-211.

Sriramulu, D. D., Lünsdorf, H., Lam, J. S. & Römling, U. (2005). Micro colony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. *J Med Microbiol* **54**, 667-676.

Stewart, W. D. P. (1973). Nitrogen fixation by photosynthetic microorganisms. *Ann Rev Microbiol* **27**, 283-316.

Stock, J. B., Ninfa, A. J. & Stock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in Bacteria. *Microbiol Rev* **53(4)**, 450-490.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalk, D. J. & other authors. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-964.

Suzuki, A. & Knaff, D. B. (2005). Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism. *Photosynth Res* **83(2)**, 191-217.

Thamdrup, B. & Dalsgaard, T. (2002). Production of N(2) through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Appl Environ Microbiol* **68(3)**, 1312-1318.

Toyofuku, M., Nomura, N., Fujii, T., Takaya, N., Maseda, H., Sawada, I., Nakajima, T. & Uchiyama, H. (2007). Quorum sensing regulates denitrification in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **189**, 4969-4972.

Ueno-Nishio, S., Mango, S., Reitzer, L. J. & Magasanik, B. (1984). Identification and regulation of the *glnL* operator-promoter of the complex *glnALG* operon of *Escherichia coli*. *J Bacteriol* **160(1)**, 379-384.

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

Van Alst, N. E., Sherrill, L. A., Iglewski, B. H. & Haidaris, C. G. (2009). Compensatory periplasmic nitrate reductase activity supports anaerobic growth of *Pseudomonas aeruginosa* PAO1 in the absence of membrane nitrate reductase. *Can J Microbiol* **55(10)**, 1133-1144.

van den Heuvel, R. H., Curti, B., Vanoni, M. A. & Mattevi, A. (2004). Glutamate synthase: a fascinating pathway from L-glutamine to L-glutamate. *Cell Mol Life Sci* **61(6)**, 669-681.

van der Oost, J., Jore, M. M., Westra, E. R., Lundgren, M. & Brouns, S. J. (2009). CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends Biochem Sci* **34**, 401-407.

van Heeswijk, W. C., Rabenberg, M., Westerhoff, H. V. & Kahn, D. (1993). The genes of the glutamine synthetase adenylylation cascade are not regulated by nitrogen in *Escherichia coli*. *Mol Microbiol* **9(3)**, 443-457.

Vander Wauven, C., Pierard, A., Kley-Raymann, M. & Haas, D. (1984). *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. *J Bacteriol* **160**, 928-934.

Vanderpool, C. K. & Gottesman, S. (2004) Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. *Mol Microbiol* **54**, 1076-1089.

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

Večerek, 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.

Ventre, I., Goodman, A. L., Filloux, A. & Lory, S. (2007). Modulation of bacterial lifestyles via two-component regulatory networks. In: Ramos J-L, Filloux A, ed. *Pseudomonas* - A model system in biology. Volume 5. Dordrecht: Springer, 311-340.

Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S. & other authors. (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci USA* **103**, 171-176.

Venturi, V. (2006). Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol Rev* **30(2)**, 274-291.

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 USA* **104(51)**, 20454-20459.

Wassarman, K. M. (2007). 6S RNA: a small RNA regulator of transcription. *Curr Opin Microbiol* **10**, 164-168.

Waters, L. S. & Storz, G. (2009). Regulatory RNAs in bacteria. *Cell* **136**, 615-628.

Wedel, A. & Kustu, S. (1995). The bacterial enhancer-binding protein NTRC is a molecular machine: ATP hydrolysis is coupled to transcriptional activation. *Genes Dev* **9(16)**, 2042-2052.

Weiss, D. S., Batut, J., Klose, K. E., Keener, J. & Kustu, S. (1991). The phosphorylated form of the enhancer-binding protein NTRC has an ATPase activity that is essential for activation of transcription. *Cell* **67(1)**, 155-167.

Weiss, V., Claverie-Martin, F. & Magasanik, B. (1992). Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. *Proc Natl Acad Sci USA* **89(11)**, 5088-5092.

Weitere, M., Bergfeld, T., Rice, S. A., Matz, C. & Kjelleberg, S. (2005). Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. *Environ Microbiol* **7(10)**, 1593-1601.

Werner, E., Roe, F., Bugnicourt, A., Franklin, M. J., Heydorn, A., Molin, S., Pitts, B. & Stewart, P. S. (2004). Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **70(10)**, 6188-6196.

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 USA* **101**, 9792-9797.

Winsor, G. L., Lam, D. K., Fleming, L., Lo, R., Whiteside, M. D., Yu, N. Y., Hancock, R. E. & Brinkman, F. S. (2011). Pseudomonas Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Res* **39(Database issue)**, D596-600.

Wood, R. E. (1976). *Pseudomonas*: the compromised host. *Hosp Pract* **11**, 91-100.

Woods, D. R. & Reid, S. J. (1993). Recent developments on the regulation and structure of glutamine synthetase enzymes from selected bacterial groups. *FEMS Microbiol Rev* **11(4)**, 273-283.

Workentine, M.-L., Chang, L., Ceri, H. & Turner, R. J. (2009). The GacS-GacA two-component regulatory system of *Pseudomonas fluorescens*: a bacterial two-hybrid analysis. *FEMS Microbiol Lett* **292**, 50-56.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., Birrer, P., Bellon, G., Berger, J. & other authors. (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* **109**, 317-325.

Wu, Q. & Stewart, V. (1998). NasFED proteins mediate assimilatory nitrate and nitrite transport in *Klebsiella oxyto*ca (*pneumoniae*) M5al. *J Bacteriol* **180(5)**, 1311-1322.

Xu, K. D., Stewart, P. S., Xia, F., Huang, C. T. & McFeters, G. A. (1998). Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl Environ Microbiol* **64(10)**, 4035-4039.

Yamashita, M. M., Almassy, R. J., Janson, C. A., Cascio, D. & Eisenberg, D. (1989). Refined atomic model of glutamine synthetase at 3.5 Å resolution. *J Biol Chem* **264(30)**, 17681-17690.

Ye, R. W., Haas, D., Ka, J. O., Krishnapillai, V., Zimmermann, A., Baird, C. & Tiedje, J. M. (1995). Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, ananalog of Fnr. *J Bacteriol* **177**, 3606-3609.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R. & other authors. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* **3**, 593-603.

Yoon, S. S., Karabulut, A. C., Lipscomb, J. D., Hennigan, R. F., Lymar, S. V., Groce, S. L., Herr, A. B., Howell, M. L., Kiley, P. J. & other authors. (2007). Two-pronged survival strategy for the major cystic fibrosis pathogen, *Pseudomonas aeruginosa*, lacking the capacity to degrade nitric oxide during anaerobic respiration. *EMBO J* **26**, 3662-3672.

Zegans, M. E., Wagner, J. C., Cady, K. C., Murphy, D. M., Hammond, J. H. & O'Toole, G. A. (2009). Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of *Pseudomonas aeruginosa*. *J Bacteriol* **191**, 210-219.

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.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533-616.

4. MANUSCRIPTS

4.1. Transcriptional regulation of nitrate assimilation in *Pseudomonas aeruginosa* occurs via transcriptional antitermination within the *nirBD*-PA1779-*cobA* operon

Bioinformatic approaches employed to analyse intergenic regions of *Pseudomonas aeruginosa* O1 (PAO1) for small RNAs (sRNA) revealed a putative RNA gene encoded upstream of the nitrate assimilation operon *nirBD-PA1779-cobA*. Here, we show that this RNA, termed NalA (*n*itrogen *a*ssimilation *l*eader A), represents the leader RNA of the *nirBD-PA1779-cobA* operon, and that *nalA* transcription is σ^{54} and NtrC dependent. A PAO1 *nalA* deletion strain and a strain bearing a deletion in ORF PA1785 failed to grow on nitrate. PA1785 was identified as a homologue of the *Azotobacter vinelandii nasT* gene, the product of which is required for transcription of the *A. vinelandii* nitrite/nitrate reductase operon. Collectively these studies revealed that transcriptional antitermination of the leader RNA NalA is required for expression of the PAO1 nitrate assimilation operon, and that this process is governed by conserved functions in PAO1 and *A. vinelandii*.

INTRODUCTION

Nitrate can be used by Bacteria as a terminal electron acceptor (nitrate respiration), to maintain the redox balance (nitrate dissimilation), and as a nutrient (nitrate assimilation). Reduction of nitrate to nitrite is a common step in all three pathways (Moreno-Vivián *et al.*, 1999).

Nitrate respiration occurs for instance in *Pseudomonas aeruginosa* biofilms colonizing the lungs of cystic fibrosis patients, where low oxygen and high nitrate concentrations favor anaerobic respiration (Yoon *et al.*, 2002; Schobert & Jahn, 2010). Three operons are involved in nitrate reduction to gaseous nitrogen (Vollack *et al.*, 1998). The *narK1K2GHJI* operon encodes the nitrate reductase required for nitrate reduction to nitrite (Schreiber *et al.*, 2007). Detoxification of nitrite to nitric oxide (NO), and further to nitrous oxide (N_2O) requires functions encoded by the *nirERS-norCB* locus (Kawasaki *et al.*, 1997). The *nosRZDFYL* operon functions (Zumft *et al.*, 1990) are finally required for reduction of nitrous oxide to inert gaseous nitrogen. The expression of all three loci occurs during anoxic conditions, and is mediated by the regulatory network composed of the regulators Anr, NarXL, Dnr and NirQ (Ye *et al.*, 1995; Arai e*t al.*, 1997; Schreiber *et al.*, 2007).

In contrast to nitrate respiration, where the nitrate reduction cascade finally results in ATP synthesis, periplasmic nitrate dissimilation mediated by the *napEFDABC* operon does not lead to energy production (Bedzyk *et al.*, 1999; van Alst *et al.*, 2009). In *Pseudomonas* spp. the periplasmic nitrate reductase is apparently used for denitrification during aerobic to anaerobic transition, as well as to maintain the redox balance by dissipating the accumulated reducing power into the reduction of nitrate to nitrite (Bedzyk *et al.*, 1999).

The Nas enzymes (for nitrate assimilation) convert inorganic nitrate to a biologically useful form *via* the reduction of nitrate to nitrite and consecutively to ammonium (Moreno-Vivián *et al.*, 1999; Richardson *et al.*, 2001). The latter step is catalysed by the siroheme-dependent NADH-nitrite reductase in various Bacteria (Lin & Stewart, 1998), including *My-*

cobacterium tuberculosis, *Klebsiella oxytoca* and *Azotobacter vinelandii* (Toukdarian & Kennedy, 1986; Cali *et al*., 1989; Malm *et al*., 2009).

In *Klebsiella spp.* expression of the nitrate assimilation (*nasFEDCBA*) operon (Fig. 1a) is subject to dual regulation. Transcription of the operon is driven by a σ^{54}-dependent promoter and mediated by phosphorylated NtrC. Full transcription further requires the nitrate-responsive NasR protein for transcription antitermination within the *nasF* leader (Lin & Stewart, 1996; Chai & Stewart, 1998). This protein possesses an N-terminal nitrate/nitrite sensing domain (Lin & Stewart, 1998; Chai & Stewart, 1998), and a C-terminal ANTAR domain (AmiR and NasR transcription antitermination regulation; Shu & Zulin, 2002) for antitermination of transcription (Lin & Stewart, 1998). Hence, regulation of nitrate assimilation is controlled by both, a general nitrogen regulation system depending on the presence of a preferred nitrogen source and by the availability of nitrate.

In *A. vinelandii* (Fig. 1a), nitrate assimilation was shown to require the nitrate and nitrite reductases NasA and NasB, respectively (Ramos *et al.*, 1993). Similarly to *Klebsiella*, the expression of the *Azotobacter* operon *nasAB* is controlled by an antitermination mechanism mediated by the antiterminator protein NasT, the activity of which is negatively controlled by the nitrate-sensitive regulator NasS (Gutierrez *et al.*, 1995; Stülke, 2002). In the absence of nitrate, transcription terminates in the *nasAB* leader as NasS inactivates NasT. In contrast, binding of nitrate to NasS seems to lead to NasT-mediated antitermination within the *nasAB* leader, and consequently to *nasAB* expression (Lin & Stewart, 1998).

Due to homologies of functions encoded in the known nitrate assimilation operons of *Klebsiella* spp. (Lin *et al.*, 1993; Wu & Stewart, 1998), *Azotobacter vinelandii* (Gutierrez et al., 1995), *Bacillus subtilis* (Ogawa *et al.*, 1995) and *Proprionibacterium freudenreichii* (Sattler *et al*., 1995), the PAO1 locus PA1778-PA1786 was inferred to encode functions required for nitrate assimilation in PAO1 (Stover *et al.,* 2000). The product of PA1779 (Fig. 1a) was later shown to display nitrate reductase activity (Noriega *et al.*, 2005). The *nirBD* genes were

inferred to encode the two subunits of nitrite reductase (Stover *et al.*, 2000; Fig. 1a) based on their homology with the *K. pneumoniae* and *K. oxytoca nasB* (Fig. 1a) and *nasC* genes, respectively (Winsor *et al.*, 2009). In contrast to *A. vinelandii* and *K. oxytoca* the molecular mechanisms underlying regulation of nitrate assimilation has not been studied in PAO1.

The PAO1 RNA NalA (*n*itrate *a*ssimilation *l*eader A) has been independently identified in this study and by Livny *et al.* (2006). We show that (i) NalA represents the leader RNA of the *nirBD-PA1779-cobA* operon, (ii) that transcription of *nalA* is driven by σ^{54} and requires NtrC, and (iii) that expression of the downstream nitrate assimilation operon requires transcriptional antitermination within the NalA leader, which is mediated by the *A. vinelandii* NasT homologue PA1785.

MATERIALS AND METHODS

Bioinformatics. A search algorithm that followed the scheme described by Lenz *et al.* (2004) was used to unravel novel sRNAs, transcription of which should be driven by a σ^{54} dependent promoter. First, the *P. aeruginosa* intergenic regions were downloaded from http://www.pseudomonas.com (Intergenic DNA, *Pseudomonas aeruginosa* PAO1, version 2004-01-14). Next, it was assumed that the putative sRNAs comprise rho-independent terminators. 524 *P. aeruginosa* rho-independent terminators were downloaded from http://www.tigr.org/software/transterm.html, of which 502 were found in intergenic regions. Using PATSER (http://liv.bmc.uu.se:16080/rsa-tools), the *P. aeruginosa* intergenic regions were then 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 taking into account that the distance between a putative sRNA start and the next ORF should be at least 50 nucleotides. Combining these 168 hits with the list of Rho-independent terminators resulted in a list of 22 intergenic loci, which contained both, a putative σ^{54}-binding site and a putative Rho-independent terminator. Among those 22 candidates only one candidate RNA emerged, in which the σ^{54}-binding site and the terminator sequences were in an appropriate sequential arrangement. The putative candidate sRNA mapped in the intergenic region between the ORFs PA1782 and PA1781 (Fig. 1a).

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani medium (Miller, 1972) supplemented with appropriate antibiotics. Antibiotics were added to final con-

centrations of 50 µg gentamycin ml^{-1}, 100 µg ampicillin ml^{-1} (*E. coli*) and 300 µg carbenicillin ml^{-1} (PAO1). Minimal salt medium (40 mM K_2HPO_4, 22 mM KH_2PO_4, 0.5 mM $MgSO_4$, 10 µM $FeSO_4$, pH 7.0) was used for gene expression analysis. Sodium succinate (20 mM) was added as a carbon source. $(NH_4)_2SO_4$, KNO_3, glutamate or glutamine were added as nitrogen sources to a final concentration of 10 mM where specified. $NaNO_2$ was added to a final concentration of 5 mM.

RNA preparation and Northern-blot analysis. Total RNA from PAO1 was purified using the hot phenol method (Lin-Chao and Bremer, 1986). The steady state levels of NalA were determined by Northern-blot analyses using 10 µg total RNA. The RNA was denatured for 5 minutes at 65°C in loading buffer containing 50% formamide, separated on 8% polyacrylamide / 8M urea gels, and then transferred to nylon membranes by semi-dry electroblotting. The RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized at 40°C with gene-specific ^{32}P-end labelled oligonucleotides (NalA, M50; 5S rRNA, I26; Supplementary Table S1) and the hybridisation signals were visualized using a PhosphoImager (Molecular Dynamics).

Determination of the 5´ and 3´ ends of NalA. Simultaneous mapping of the 5´ and 3´ ends of NalA RNA was performed as follows. Total RNA was purified from PAO1(pME*nalA*) (Table 1) grown in LB medium to an OD_{600} of 2.5. The RNA was treated with tobacco alkaline phosphatase (TAP) (Epicentre Biotechnologies), which removes 5´-pyrophosphates. The 5´ and 3´ ends of the TAP treated RNA were ligated using T4 RNA ligase (Fermentas). First strand cDNA, second strand synthesis and final product amplification were performed using the OneStep RT-PCR Kit (Qiagen) and 250 ng of total RNA. The primer used for first strand cDNA synthesis was M50. Primer N49 (Supplementary Table S1) was added after first strand cDNA synthesis in addition to primer M50. A non-TAP-treated, non-ligated RNA was used as a negative control. The reaction products were loaded on a 12% polyacrylamide gel, the

expected product was sliced out of the gel, eluted and finally cloned into the pGEM T-easy vector (Promega) using standard procedures. The inserts of several plasmid clones were sequenced to reveal the 5´-3´ junction.

Construction of the PAO1 *nalA* deletion strain. The sequence upstream (bp -691 to -5 with regard to the transcriptional start of *nalA*) and the sequence downstream (bp +63 to +814) of *nalA* were amplified by PCR using the oligonucleotide pairs L49/M49 and N49/O49 (Supplementary Table S1), respectively, and chromosomal PAO1 DNA as template. The 752 bp downstream fragment was cloned into the *Pvu*I and *Eco*RI site of plasmid pSUP202, resulting in pSUPΔ*nalA*down. The 687 bp upstream fragment was cloned into the *Eco*RI and *Nco*I site of pSUPΔ*nalA*down, resulting in pSUPΔ*nalA*. Deletion of *nalA* in the plasmid was confirmed by DNA sequencing (Supplementary Fig. S1a), and pSUPΔ*nalA* was subsequently transformed into *E. coli* S17-1 and transferred by conjugation to PAO1. In order to ensure that a double crossover had occurred we scored for tetracycline sensitivity of the PAO1Δ*nalA* mutant. The 68 nucleotides chromosomal deletion (Supplementary Fig. S1a) spanning nucleotides -5 to +63 of NalA (the transcriptional terminator comprising nucleotides +65 to +106 was retained) was further confirmed by PCR using genomic DNA from PAO1Δ*nalA* and primer pair U46/I47 (Supplementary Table S1) followed by sequencing of the obtained PCR product.

Construction of plasmids and site-directed mutagenesis. All cloning procedures were carried out in *E. coli* strain Top10 and the relevant DNA segments of all newly constructed plasmids were verified by DNA sequencing.

For NalA overproduction, plasmid pME*nalA* was constructed. The *nalA* fragment (comprising the PAO1 genome sequence 1928837 – 1928656; Winsor *et al*., 2009) was amplified by PCR using PAO1 chromosomal DNA together with the forward primer I29 con-

taining a *Xba*I site, and the reverse primer J29 containing an *Eco*RI site (Supplementary Table S1). The obtained 205 nucleotides long fragment was inserted into the corresponding sites of plasmid pME4510, giving rise to pME*nalA*.

For construction of the transcriptional p*nalA´-lacZ* fusion, the region comprising bp -231 to + 33 with regard to the transcriptional start of NalA was amplified by PCR using PAO1 genomic DNA together with forward primer U46 containing a *Sal*I site and reverse primer V46 (Supplementary Table S1) containing a *Bam*HI site. The PCR product was cloned into the corresponding sites of plasmid pQF50, resulting in pQF*nalA*.

Mutations at the -24 and the -12 site, respectively, of the putative σ^{54} *nalA* promoter were introduced by PCR amplification of 1 ng pQF*nalA* plasmid using either the mutagenic primer M57 (-12 mutations) or O63 (-24 mutations) together with primer V46 (Supplementary Table S1). The amplified linear pQF12mut*nalA* and pQF24mut*nalA* plasmids were circularized using T4 DNA ligase (Fermentas) and transformed into *E. coli* Top10. Background contamination by template plasmid was reduced by treatment with 10 U *Dpn*I prior to transformation. The -12 and -24 mutations in the NalA promoter present in the respective plasmids were confirmed by DNA sequencing (Supplementary Fig. S1b).

The two putative NtrC binding sites upstream of *nalA* (-128 bp to -113 and -108 to -93 bp with regard to *nalA* transcriptional start) were deleted by amplification of 1 ng pQF*nalA* plasmid using the oligonucleotides A81/L80 (Supplementary Table S1). The amplified linear plasmid pQFΔNtrCBS*nalA* was subsequently circularized with T4 DNA polymerase. Background contamination by template plasmid was reduced by treatment with 10 U *Dpn*I prior to transformation. The correct deletion of the putative NtrC binding sites was confirmed by DNA sequencing (Supplementary Fig. S1b).

For the transcriptional antitermination studies, the PAO1 genome segment corresponding to bp 1928991 - 1928592 (Winsor *et al*., 2009) encompassing the *nalA* promoter, *nalA* or the *nalA* deletion and the first 12 codons of *nirB* (bp +1 to +36 with respect to the A of the *nirB* start codon) was PCR amplified using genomic DNA derived from PAO1 or from PAO1Δ*nalA* together with primer pair U46/R64. The obtained fragments (410 bp for p*nalA-nirB* and 342 bp for pΔ*nalA-nirB*) were inserted into the *Sal*I and *Bam*HI sites of plasmid pQF50, resulting in pQF*nalA-nirB* and pQFΔ*nalA-nirB*, respectively.

β-Galactosidase assay. The PAO1 strains were grown in minimal medium containing succinate (20 mM) and ammonium (10 mM) under aeration at 37°C until they reached an OD_{600nm} of 0.8. The cells were washed in PBS (140 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and then either resuspended in fresh nitrogen-free minimal medium containing succinate (nitrogen depletion), or in succinate containing minimal medium in the presence of the indicated nitrogen source. Samples were withdrawn when they reached and OD_{600nm} of 1 or at the indicated time (hours) after shift to fresh medium. All experiments were done in triplicate. The β-galactosidase values were determined as described (Miller, 1972).

RT-PCR. PAO1 was grown for 18 hours in minimal medium containing succinate (20 mM) and ammonium (10 mM). The cells were washed in PBS and resuspended in nitrogen-free minimal medium containing succinate (BM2) or supplemented with nitrate where indicated. Samples were withdrawn 5 hours following the shift to fresh medium. Purified total RNA (10 µg) was treated with 20 U RNase-free DNase I (Roche). For cDNA synthesis, 20 pmol of each of the primers N55/R64 (Supplementary Table S1) were annealed to 5 µg RNA for 5 min at 65 °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 of AMV reverse transcriptase (Promega) were added, and the reaction was continued for 1 h at 50 °C. From this

reaction 2 µl aliquots were used as templates in 25 µl PCR amplification reactions using GoTaq Green Master Mix (Promega) and the primer pairs N55/R64 or Q64/R64 (Supplementary Table S1) at a final concentration of 0.5 pmol each. The generated PCR fragments were analyzed on 6% polyacrylamide gels. 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.

RESULTS AND DISCUSSION

The NalA RNA represents the leader of the PAO1 *nirBD*-PA1779-*cobA* operon

As described in Materials and Methods the bioinformatic search for sRNA candidates with a σ^{54}–dependent promoter predicted a small RNA gene upstream of the PAO1 *nirB* gene (Fig. 1a). The same sRNA candidate was previously identified as P11 in a study by Livny *et al.* (2006). Synthesis of the candidate RNA was verified by Northern-blot analysis (Livny *et al.*, 2006; Fig. 1b). Given the genomic location of *p11* upstream of the PAO1 *nirBD*-PA1779-*cobA* operon (Fig. 1a), we re-named P11 as NalA (*n*itrogen *a*ssimilation *l*eader A). The predicted secondary structure contains 3 putative stem-loop structures (Supplementary Fig. S2a), the distal one of which is followed by a string of U´s (T´s in Fig. 1c), which is a hallmark of rho independent transcriptional terminators (Platt, 1986).

NalA transcription is dependent on σ^{54} and NtrC

NalA was initially detected in stationary phase (Livny *et al.*, 2006). Therefore, total RNA purified from PAO1 grown to stationary phase was used to map the 5´and 3´ends of NalA as outlined in Materials and Methods. This analysis (Fig. 1c; Supplementary Fig. S3) revealed that NalA is 96 nucleotides in length. Upstream of the NalA start, putative -12 (CT**GC**TT) and -24 (TT**GG**CA) sequence motifs (Fig. 1c) were found that are typical signatures for σ^{54} -dependent promoters (Morett & Buck, 1989; consensus: TGG-N_{10}-GCT). In addition, two putative binding sites for the response regulator NtrC (Ames & Nikaido, 1985; Hirschman *et al.*, 1985; MacFarlane & Merrick, 1985) were identified upstream of the putative σ^{54} promoter (Fig. 1c). Similarly, two NtrC binding sites have been previously identified upstream of the *K. oxytoca* nitrate assimilation operon (Chai & Stewart, 1999; Supplementary Figure S2b).

First, we tested whether σ^{54} is required for NalA synthesis. A transcriptional fusion comprising the putative σ^{54}-dependent *nalA* promoter abutted with the *lacZ* gene (p*nalA´-lacZ*) was constructed and the promoter activity was monitored by measuring the β-galactosidase activity in PAO1 and the σ^{54} deficient mutant PAO1*ΔrpoN*. As shown in Fig. 2a, the ß-galactosidase activity was very low in the PAO1*ΔrpoN* strain, whereas a strong increase was observed in PAO1 after 4 or 21 hours after shift to nitrogen-free minimal medium. The *in vivo* assays were mirrored by Northern blot analyses, revealing a strong increase of the steady levels of NalA 21 hours after nitrogen depletion (Supplementary Fig. S4a).

As binding of σ^{54} to its target promoter results in the formation of a transcriptionally inactive closed complex, the response regulator of two component systems is usually required for transcriptional activation of the downstream gene (Kustu *et al*., 1989; Merrick, 1993; Wei & Chung-Dar, 2007; Hervás *et al*., 2009). Given the presence of putative NtrC binding sites upstream of the *nalA* start (Fig. 1c), we next asked whether the response regulator NtrC is required for activation of the σ^{54}–dependent *nalA* promoter. As shown in Fig. 2b, the differential β-galactosidase activities observed with PAO1 and the PAO1Δ*ntrC* strain strongly suggested that the σ^{54} dependent *nalA* promoter requires NtrC for activation.

The σ^{54} and NtrC dependence of the *nalA* promoter was further verified by introducing mutations in the -12 and -24 regions of the putative σ^{54} promoter and by deletion of the two putative NtrC binding sites (Fig. 1c; Supplementary Fig. S1b). Both modifications, in either the -12 or the -24 region, abrogated promoter activation 4 hours after nitrogen depletion. Similarly, deletion of the NtrC binding sites resulted in a significant decrease of the promoter activity (Fig. 2c).

In *Pseudomonas putida*, open complex formation from σ^{54}-dependent promoters strictly depends on phosphorylated NtrC (Hervás *et al.*, 2009). Although non-phosphorylated NtrC is still capable of binding to the *P. putida glnK* promoter, only phosphorylated NtrC activated

transcription of *glnK* (Hervás *et al.*, 2010). The phosphorylation status of NtrC depends on the nitrogen source; it is hardly phosphorylated in the presence of ammonium and glutamine, whereas it is phosphorylated in the presence of glutamate, nitrate and nitrite (Atkinson *et al.*, 1994; Kamberov *et al.*, 1995). We therefore monitored the β-galactosidase activity conferred by the p*nalA´-lacZ* transcriptional fusion upon growth in different nitrogen sources. As anticipated, PAO1 grown in BM2 minimal medium supplemented with ammonium or glutamine, which are favoured nitrogen sources for PAO1 (Potts & Clarke, 1976; Janssen *et al.*, 1981), displayed a very low promoter activity (Supplementary Fig. S4b). In contrast, PAO1 grown in the presence of glutamate, nitrate or nitrite as sole nitrogen source showed an increase in the transcriptional activity of the p*nalA* promoter (Supplementary Fig. S4b). Taken together, these studies revealed that *nalA* transcription is mediated by a σ^{54}– and NtrC- dependent promoter.

NalA and nitrate are required for transcription of genes located downstream of *nalA*

As mentioned above, expression of the nitrate-nitrite assimilation operons in *K. oxytoca* and *A. vinelandii* occurs through transcriptional antitermination within the operon leader. Given the similarity in the genetic set up of the nitrate assimilation operons of *P. aeruginosa* and *A. vinelandii* (Fig. 1a), and the similarity of the *nalA* sequence with the proximal sequences of *K. oxytoca nasF* and *A. vinelandii nasA*, respectively (Fig. 3), we next tested whether expression of the PAO1 nitrate assimilation operon requires antitermination within the *nalA* coding sequence. RT-PCR analysis of total RNA purified from PAO1 grown in BM2 minimal medium with or without nitrate revealed that co-transcription of *nalA* and *nirB* occurred only in the presence of nitrate (Fig. 4a). Moreover, in the presence of nitrate β-galactosidase synthesis was only conferred by plasmid pQF*nalA-nirB* comprising the *nalA* promoter, *nalA* and the first 12 codons of *nirB* abutted with the *lacZ* gene, but not with the variant plasmid pQFΔ*nalA-nirB* comprising the *nalA* deletion (Fig. 4b). Similarly, the PAO1Δ*nalA* strain

failed to grow on nitrate as sole nitrogen source, whereas PAO1 and the PAO1Δ*nalA* strain grew comparably in the presence of ammonium (Fig. 5a). Taken together, these data suggested that transcription of the PAO1 nitrate assimilation operon depends on antitermination within *nalA*.

The *A. vinelandii* NasT homologue PA1785 is required for nitrate assimilation

In *K. oxytoca*, antitermination occurs through binding of the antiterminator protein NasR to the upper portion of the 1:2 stem-loop structure of the *nasF* leader RNA (Chai and Stewart, 1999). In the PAO1Δ*nalA* mutant, which failed to grow on nitrate, the corresponding sequence required for NasR binding in *K. oxytoca* was deleted (Fig. 3), but the rho-independent terminator was retained. Thus, the absence of the probable binding sequence for a putative PAO1 antiterminator protein but the retention of the terminator could readily explain the lack of PAO1Δ*nalA* to grow with nitrate (Fig. 5a). Similarly to *K. oxytoca***,** transcriptional antitermination of the *A. vinelandii nasAB* operon requires the RNA-binding protein NasT (Gutierrez *et al.*, 1995). These findings together with the studies described above prompted us to search for an *A. vinelandii* NasT homologue in PAO1. The product encoded by PA1785 showed 96% similarity with the *A. vinelandii* NasT protein (Supplementary Fig. S5a). In addition, *Pfam* (Bateman *et al.,* 2000) predicted both proteins to contain an ANTAR motif (AmiR and NasR Transcriptional Antitermination Regulators; Supplementary Fig. S5a), which is was shown to be required for RNA binding and for antitermination of transcription by *K. oxytoca* NasR (Shu & Zhulin, 2002). Therefore, we tested whether the PA1875 product is necessary for utilization of nitrate as the sole nitrogen source. As shown in Fig. 5b, the insertional inactivation of PA1875 in strain PW4148 resulted in cessation of growth upon shift to nitrate-containing minimal medium.

In contrast to the *K. oxytoc*a NasR protein, which apparently contains a domain for nitrate recognition and for antitermination (Goldman *et al.*, 1994; Lin & Stewart, 1998), the NasT protein of *A. vinelandii* lacks the nitrate sensor domain (Gutierrez *et al.*, 1995). However, the NasS protein of *A. vinelandii*, which binds nitrate (Gutierrez *et al.*, 1995), shares similarities with the *P. aeruginosa* AmiC protein (Gutierrez *et al.*, 1995). The AmiC protein is known to bind to and to sequester the AmiR protein in the absence of the ligand amide (Wilson *et al.*, 1996), thereby inhibiting AmiR-mediated antitermination of the *amiEBCRS* operon. In analogy, Lin & Stewart (1998) hypothesized that in *A. vinelandii* NasT is sequestered by NasS in the absence of nitrate, whereas nitrate binding to NasS relieves NasT inhibition and allows NasT to act as an antiterminator. We found 76% homology between PA1786 and the *A. vinelandii* NasS protein using Clustal analysis (Larkin *et al.*, 2007; Goujon *et al.*, 2010; Supplementary Fig. S5b). Given the homologies between the NasS/T proteins of both *A. vinelandii and P. aeruginosa*, we therefore suggest to rename the PAO1 ORFs 1785 and 1786 as *nasT* and *nasS*, respectively (Fig. 1a). In summary, this study strongly suggests that nitrate assimilation in PAO1 requires antitermination in the *nalA* leader when nitrate is present as a sole nitrogen source.

The phylogenetic relationships between several *Pseudomonas* spp. and *A. vinelandii* for several housekeeping functions revealed that the *A. vinelandii* proteins were most closely related to the corresponding PAO1 orthologues. Therefore, Rediers *et al.* (2004) suggested that *A. vinelandii* belongs to the genus *Pseudomonas*. It is therefore not surprising that nitrate assimilation is governed in PAO1 and *A. vinelandii* by conserved *cis*-elements and *trans*-acting factors (Fig. 6). At variance, although nitrate assimilation in *Klebsiella* spp. is as well regulated by antitermination, the overall gene organization of the locus and the antitermination protein involved are distinct from PAO1 and *A. vinelandii* (Fig. 1a).

Finally, as NalA was first described as a putative sRNA (Livny *et al.*, 2006), it seems worth noting that every identified bacterial sRNA candidate should be first scrutinized towards the possibility to function in *cis,* i.e. whether they are involved in transcriptional termination /antitermination or whether they are part of riboswitches.

ACKNOWLEDGEMENTS

We are grateful to Dr. Dieter Haas from the Département de Microbiologie Fondamentale at the Université de Lausanne (Switzerland) and C. Manoil from the Genome Science University of Washington (USA) for providing strains. This work was supported by grant AF04311 and by the doctoral program "RNA Biology" (W-1207) from the Austrian Science Fund.

REFERENCES

Ames, G. F. - L. & Nikaido, K. (1985). Nitrogen regulation in *Salmonella typhimurium*. Identification of an *ntrC* protein-binding site and definition of a consensus binding sequence. *EMBO J* **4**, 539-547.

Arai, H., Kodama, T. & Igarashi, Y. (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)**, 1141-1148.

Atkinson, M. R., Kamberov, E. S., Weiss, R. L., & Ninfa, A. J. (1994). Reversible uridylylation of the *Escherichia coli* P_{II} signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC). *J Biol Chem* **269**, 28288-28293.

Bateman, A., Birney, E., Durbin, R., Eddy, S. R., Howe, K. L. & Sonnhammer, E. L. L. (2000). The Pfam protein families database. *Nucleic Acids Res* **28**, 263-266.

Bedzyk, L., Wang, T. & Ye, R. W. (1999). The periplasmic nitrate reductase in *Pseudomonas* sp. strain G-179 catalyses the first step of denitrification. *J Bacteriol* **181(9)**, 2802-2806.

Cali, B. M., Micca, J. L. & Stewart, V. (1989). Genetic regulation of nitrate assimilation in *Klebsiella oxytoca* M5al. *J Bacteriol* **171(5)**, 2666-2672.

Chai, W. & Stewart, V. (1998). NasR, a novel RNA-binding protein, mediates nitrate responsive transcription antitermination of the *Klebsiella oxytoca* M5al *nasF* operon leader *in vitro*. *J Mol Biol* **283**, 339-351.

Chai, W. & Stewart, V. (1999). RNA sequence requirements for NasR-mediated, nitrate-responsive transcription antitermination of the *Klebsiella oxytoca* M5a1 *nasF* operon leader. *J Mol Microbiol* **292**, 203-216.

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

Farinha, M. A. & Kropinski, A. M. (1990). Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J Bacteriol* **172(6)**, 3496-3499.

Goldman, B. S., Lin, J. T. & Stewart, V. (1994). Identification and structure of the *nasR* gene encoding a nitrate- and nitrite-responsive positive regulator of *nasFEDCBA* (nitrate assimilation) operon expression in *Klebsiella pneumoniae* M5al. *J Bacteriol* **176(16)**, 5077-5085.

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J. & Lopez, R. (2010). A new bioinformatics analysis tools framework at EMBL-EBI. *Nucl Acids Res* **38**, 695-699.

Gutierrez, J. C., Ramos, F., Ortner, L. & Tortolero, M. (1995). *nasST*, two genes involved in the induction of the assimilatory nitrite-nitrate reductase operon (*nasAB*) of *Azotobacter vinelandii*. *Mol Microbiol* **18(3)**, 579-591.

Hervás, A. B., Canosa, I. & Santero, E. (2010). Regulation of glutamate dehydrogenase in *Pseudomonas putida* results from its direct repression by NtrC under nitrogen-limiting conditions. *Mol Microbiol* **78(2)**, 305-319.

Hervás, A. B., Canosa, I., Little, R., Dickson, R. & Santero, E. (2009). NtrC-dependent regulatory network for nitrogen assimilation in *Pseudomonas putida*. *J Bacteriol* **191(19)**, 6123-6135.

Heurlier, K., Denervaud, V., Pessi, G., Reimmann, C. & Haas, D. (2003). Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185(7)**, 2227-2235.

Hirschman, J., Wong, P. -K., Sei, K., Keener, J. & Kustu, S. (1985). Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric Bacteria activate *ginA* transcription *in vitro*: Evidence that the *ntrA* product is a sigma factor. *Proc Natl Acad Sci USA* **82**, 7525-7529.

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

Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C. & other authors (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100(24)**, 14339-14344.

Janssen, D. B., Herst, P. M., Joosten, H. M. L. J. & Drift, C. (1981). Nitrogen control in *Pseudomonas aeruginosa*: a role for glutamine in the regulation of the synthesis of NADP-dependent glutamate dehydrogenase, urease and histidase. *Arch Microbiol* **128(4)**, 398-402.

Kamberov, E. S., Atkinson, M. R. & Ninfa, A. J. (1995). The *Escherichia coli* P_{II} signal transduction protein is activated upon binding 2-ketoglutarate and ATP. *J Biol Chem* **270**, 17797-17807.

Kawasaki, S., Arai, H., Kodama, T. & Igarashi, Y. (1997). Gene cluster for dissimilatory nitrite reductase (*nir*) from *Pseudomonas aeruginosa*: sequencing and identification of a locus for heme d1 biosynthesis. *J Bacteriol* **179(1)**, 235-242.

Kustu, S., Santero, E., Keenes, J., Popham, D. & Weiss, D. (1989). Expression of σ^{54} (NtrA) – dependent genes is probably united by a common mechanism. *Microbiol Reviews* **53(3)**, 367-376.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). ClustalW and ClustalX version 2. *Bioinformatics* **23(21)**, 2947-2948.

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(1)**, 69-82.

Lin, J. T., Goldman, B. S. & Stewart, V. (1993). Structures of genes *nasA* and *nasB*, encoding assimilatory nitrate and nitrite reductases in *Klebsiella pneumoniae* M5al. *J Bacteriol* **175(8)**, 2370-2378.

Lin, J. T. & Stewart, V. (1996). Nitrate and nitrite-mediated transcription antitermination control of *nasF* (nitrate assimilation) operon expression in *Klebsiella oxytoca* M5al. *J Mol Biol* **256(3)**, 423-435.

Lin, J. T. & Stewart, V. (1998). Nitrate assimilation by Bacteria. *Adv Microbiol Physiol* **39**, 1-30.

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. *Nucl Acids Res* **34(12)**, 3484-3493.

MacFarlane, S. A. & Merrick, M. (1985). The nucleotide sequence of the nitrogen regulation gene *ntrR* and the *glnA–nirRC* intergenic region of *Klebsiella oxytoca*. *Nucleic Acids Res* **13**, 7591-7605.

Malm, S., Tiffert, Y., Micklinghoff, J., Schultze, S., Joost, I., Weber, I., Horst, S., Ackermann, B., Schmidt, M. & other authors (2009). The roles of the nitrate reductase NarGHJI, the nitrite reductase NirBD and the response regulator GlnR in nitrate assimilation of *Mycobacterium tuberculosis.* *Microbiology* **155**, 1332-1339.

Merrick, M. J. (1993). In a class of its own – The RNA polymerase sigma factor σ^{54} (σ^{N}). *Mol Microbiol* **10(5)**, 903-909.

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

Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R. & Castillo, F. (1999). Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. *J Bacteriol* **181(21)**, 6573-6584.

Morett, E. & Buck, M. (1989). *In vivo* studies on the interaction of RNA polymerase-σ^{54} with the *Klebsiella oxytoca* and *Rhizobium meliloti nifH* promoters: the role of NifA in the formation of an open promoter complex. *J Mol Biol* **210(1)**, 65-77.

Noriega, C., Hassett, D. J. & Rowe, J. J. (2005). The *mobA* gene is required for assimilatory and respiratory nitrate reduction but not xanthine dehydrogenase activity in *Pseudomonas aeruginosa*. *Curr Microbiol* **51**, 419-424.

Ogawa, K., Akagawa, E., Yamane, K., Sun, Z. W., LaCelle, M., Zuber, P. & Nakano, M. M. (1995). The *nasB* operon and *nasA* gene are required for nitrate/nitrite assimilation in *Bacillus subtilis*. *J Bacteriol* **177(5)**, 1409-1413.

Platt, T. (1986). Transcription termination and the regulation of gene expression. *Ann Rev Biochem* **55**, 339-372.

Potts, J. R. & Clarke, P. H. (1976). The effect of nitrogen limitation on catabolite repression of amidase, histidase and urocanase in *Pseudomonas aeruginosa*. *J Gen Microbiol* **93**, 377-387.

Ramos, F., Blanco, G., Gutiérrez, J. C., Luque, F. & Tortolero, M. (1993). Identification of an operon involved in the assimilatory nitrate-reducing system of *Azotobacter vinelandii*. *Mol Microbiol* **8(6)**, 1145-1153.

Rediers, H., Vanderleyden, J. & de Mot, R. (2004). *Azotobacter vinelandii:* a *Pseudomonas* in disguise? *Microbiol* **150,** 1117-1119.

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S. & Taylor, C. J. (2001). Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell Mol Life Sci* **58(2)**, 165-178.

Rist, M. & Kertesz, M. A. (1998). Construction of improved vectors for promoter characterisation in *Pseudomonas aeruginosa* and other Gram-negative Bacteria. *FEMS Microbiol Letters* **169**, 179-183.

Sattler, I., Roessner, C. A., Stolowich, N. J., Hardin, S. H., Harris-Haller, L. W., Yokubaitis, N. T., Murooka, Y., Hashimoto, Y. & Scott, A. I. (1995). Cloning, sequencing, and expression of the uroporphyrinogen III methyltransferase *cobA* gene of *Propionibacterium freudenreichii* (*shermanii*). *J Bacteriol* **177(6)**, 1564-1569.

Schobert, M. & Jahn, D. (2010). Anaerobic physiology of *Pseudomonas aeruginosa* in the cystic fibrosis lung. *Int J Med Microbiol* **300(8)**, 549-556.

Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M. & Jahn, D. (2007). The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol* **189(11)**, 4310-4314.

Setubal, J. C., dos Santos, P., Goldman, B. S., Ertesvåg, H., Espin, G., Rubio, L. M., Valla, S., Almeida, N. F., Balasubramanian, D. & other authors (2009). Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. *J Bacteriol* **191(14)**, 4534-4545.

Shu, C. J. & Zhulin, I. B. (2002). *ANTAR:* an RNA-binding domain in transcription antitermination regulatory proteins. *Trends Bioch Sci* **27(1)**, 3-5.

Simon, R., O'Connell, M., Labes, M. & Pühler, A. (1986). Plasmid vectors for the genetic analysis and manipulation of rhizobia and other Gram-negative Bacteria. *Methods Enzymol* **118**, 640-645.

Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Nat Biotechnol* **1**, 784-791.

Sonnleitner, E., Sorger-Domenigg, T., Madej, M. J., Findeiss, S., Hackermüller, J., Hüttenhofer, A., Stadler, P. F., Bläsi, U. & Moll, I. (2008). Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. *Microbiology* **154**, 3175-3187.

Stover, K. C., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J. & other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1: an opportunistic pathogen. *Nature* **406**, 959-964.

Stülke, J. (2002). Control of transcription termination in Bacteria by RNA-binding proteins that modulate RNA structures. *Arch Microbiol* **117**, 433-440.

Toukdarian, A. & Kennedy, C. (1986). Regulation of nitrogen metabolism in *Azotobacter vinelandii*: isolation of *ntr* and *glnA* genes and construction of *ntr* mutants. *EMBO J* **5(2)**, 399-407.

Van Alst, N. E., Sherrill, L. A., Iglewski, B. H. & Haidaris, C. G. (2009). Compensatory periplasmic nitrate reductase activity supports anaerobic growth of *Pseudomonas aeruginosa* PAO1 in the absence of membrane nitrate reductase. *Can J Microbiol* **55(10)**, 1133-1144.

Vollack, K. - U., Xie, J., Härtig, E., Römling, U. & Zumft, W. G. (1998). Localisation of denitrification genes on the chromosomal map of *Pseudomonas aeruginosa*. *Microbiology* **144**, 441-448.

Wei, L. & Chung-Dar, L. (2007). Regulation of carbon and nitrogen utilisation by CbrAB and NtrBC two component systems in *Pseudomonas aeruginosa*. *J Bacteriol* **189(15)**, 5413-5420.

Wilson, S. A., Wachira, S. J., Norman, R. A., Pearl, L. H. & Drew, R. E. (1996). Transcription antitermination regulation of the *Pseudomonas aeruginosa* amidase operon. *EMBO J* **15(21)**, 5907-5916.

Winsor, G. L., Van Rossum, T., Lo, R., Khaira, B., Whiteside, M. D., Hancock, R. E. & Brinkman, F. S. (2009). Pseudomonas Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. *Nucleic Acids Res* **37**, 483-488.

Wu, Q. & Stewart, V. (1998). NasFED proteins mediate assimilatory nitrate and nitrite transport in *Klebsiella oxytoca* (*pneumoniae*) M5al. *J Bacteriol* **180(5)**, 1311-1322.

Ye, R. W., Haas, D., Ka, J. O., Krishnapillai, V., Zimmermann, A., Baird, C. & Tiedje, J. M. (1995). Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J Bacteriol* **177(12)**, 3606-3609.

Yoon, S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H., DeKievit, T., Gardner, P. R. & other authors (2002). Development and persistence of anaerobic *Pseudomonas aeruginosa* biofilms: relevance to pathogenesis and therapy of cystic fibrosis lung disease. *Dev Cell* **3**, 593-603.

Zumft, W. G., Viebrock-Sambale, A. & Braun, C. (1990). Nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*. Genes for copper-processing and properties of the deduced products, including a new member of the family of ATP/GTP-binding proteins. *Eur J Biochem* **192(3)**, 591-599.

Table 1. Strains and plasmids used in this study

Strain or Plasmid	Genotype/Relevant features	Reference or Origin
***P. aeruginosa* strains**		
PAO1	Wild-type	Holloway *et al.* (1979)
PAO6358	PAO1Δ*rpoN*	Heurlier *et al.* (2003)
PAO1*ΔnalA*	*nalA* deletion strain	This study
PAO6743	PAO1 *ntrC*::Gm^R	Haas Laboratory
PW4148	PA1785-H09::IS*lacZ*/hah	Jacobs *et al.* (2003)
***E. coli* strains**		
Top 10	F-, *mcrA*, D(*mrr-hsd*RMS-*mcr*BC),p80*lacZ*DM15 D*lacX*74, *deoR*, *recA1*, *araD*139D(*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		
pME4510	Broad-host range promoter-probe plasmid, Gm^R	Rist *et al.* (1998)
pME*nalA*	pME4510 harbouring *nalA* (authentic promoter), Gm^R	Sonnleitner *et al.* (2008)
pGEM T-easy vector	Vector designed for TA-cloning, Amp^R	Promega
pQF50	Promoterless cloning vector for transcriptional *lacZ* fusions, Amp^R	Farinha and Kropinski (1990)
pQF*nalA*	Transcriptional *nalA-lacZ* fusion, Amp^R	This study
pQF12mut*nalA*	pQF*nalA* with GC → TA mutation in the -12 box of σ^{54} promoter, Amp^R	This study
pQF24mut*nalA*	pQF*nalA* with GG → AA mutation in the -24 box of σ^{54} promoter, Amp^R	This study
pQFNtrCBS*nalA*	pQF*nalA* with deletion in the two GCACC -N_5- GGTGC binding sites for NtrC, Amp^R	This study
pSUP202	pBR325 derived suicide vector; *mob*, Tc^R, Amp^R, Cm^R	Simon *et al.* (1983)
pSUP*ΔnalA*	pSUP202 with deletion in *nalA* (position - 5 to + 63 with regard to the transcriptional start site), Tc^R	This study
pQF*nalA-nirB*	Transcriptional *nalA-nirB-lacZ* fusion (containing the first 12 *nirB* codons), Amp^R	This study
pQF*ΔnalA-nirB*	pQF*nalA-nirB* with deletion in *nalA* from position - 5 to + 63 (with regard to the transcriptional start site), Amp^R	This study

FIGURE LEGENDS

Fig. 1. (a) Genetic organization of the nitrite/nitrate assimilation operon genes in *K. oxytoca* (Lin & Stewart, 1996), *A. vinelandii* (Setubal *et al.*, 2009) and PAO1 (Winsor *et al.*, 2009). ORFs encoding functional classes of proteins are color coded; nitrate-sensor and antiterminator proteins are depicted by orange arrows, nitrate transporters are depicted by green arrows and nitrate and nitrite reductases are depicted by light blue and dark blue arrows, respectively. The leader sequences of the nitrate and nitrite reductase genes and transcriptional terminators within these sequences (circle) are shown in red. (b) Detection of the ~ 100 nucleotides NalA RNA by Northern-blot analysis as described in Materials and Methods. RNA markers (M) are shown at the left. (c) Promoter sequence of *nalA*. The putative -24 and -12 boxes of the σ^{54} dependent promoter are shown in bold. The σ^{54} consensus sequence is shown above the sequence. Mutations introduced in the -24 and -12 regions are indicated. The putative palindromic NtrC binding motifs are shown by horizontal arrows. The NtrC consensus sequences (Ames & Nikaido, 1985) are shown above the sequence. The introduced deletion of the NtrC binding sites is indicated in light grey. The transcriptional start site of *nalA* is underlined and its 5´and the 3´ends are marked by vertical arrows. The *nalA* terminator sequence is denoted by horizontal arrows. The position within *nalA* used for the transcriptional fusion with *lacZ* is indicated.

Fig. 2. NalA expression is dependent on σ^{54} and NtrC upon nitrogen depletion. The strains were grown as described in the text and the β-galactosidase activity conferred by plasmid pQF*nalA* in PAO1 and PAO1*ΔrpoN* (a) and PAO1 and PAO1Δ*ntrC* (b), respectively, was determined four hours after nitrogen depletion. (c) Mutations in the -24 and -12 motifs (Fig. 1c) of the σ^{54} dependent promoter abolished promoter activation upon nitrogen depletion. The deletion of the NtrC binding regions upstream of *nalA* (Fig 1c) resulted in decreased promoter activity. All experiments were performed in triplicate. Error bars, SD.

Fig. 3. Sequence similarity of the leader sequences preceding *nasF* (*K. oxytoca* (Kox); Chai & Stewart, 1999), *nirB* (PAO1 (Pae); Stover *et al.*, 2000) and *nasA* (*A. vinelandii* (Avin); Setubal *et al.*, 2009) revealed by CLUSTAL 2.0.12 multiple sequence alignment analysis (Larkin *et al.*, 2007; Goujon *et al.*, 2010). The segment of the *K. oxytoca nasF* leader required for interaction with the antiterminator protein NasR is shown in red (Chai and Stewart, 1999).

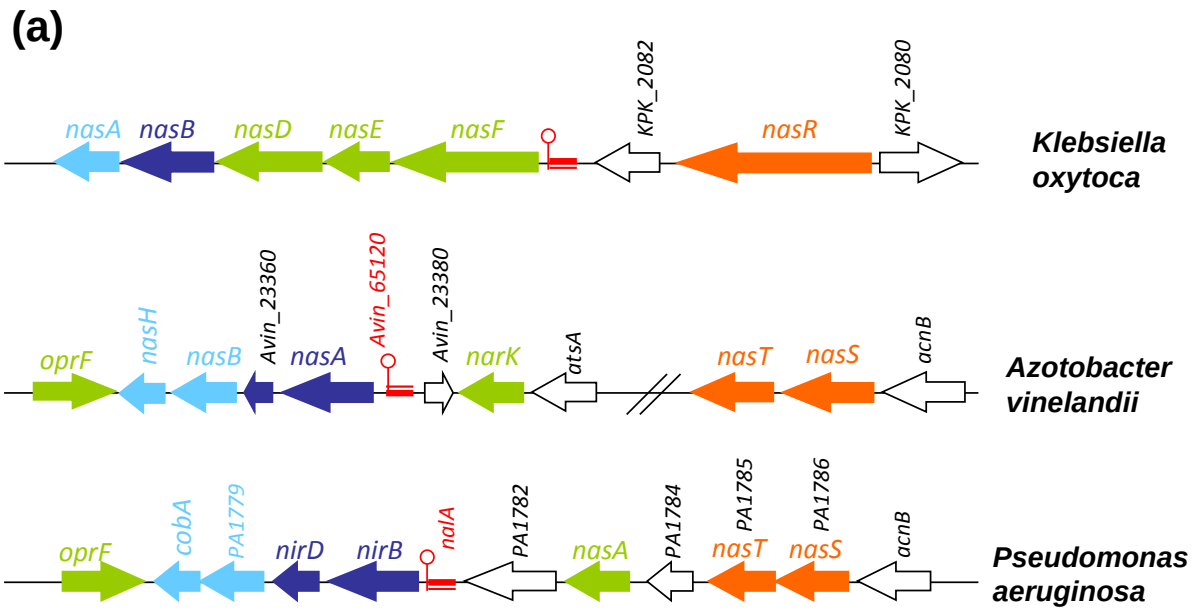
The sequence embedded in red brackets was deleted in the PAO1Δ*nalA* mutant strain. The asterisks (*) indicates positions with exact nucleotide match. The colons (:) indicates positions with conserved nucleotide substitutions. The points (.) indicate positions with semi-conserved nucleotide substitutions.

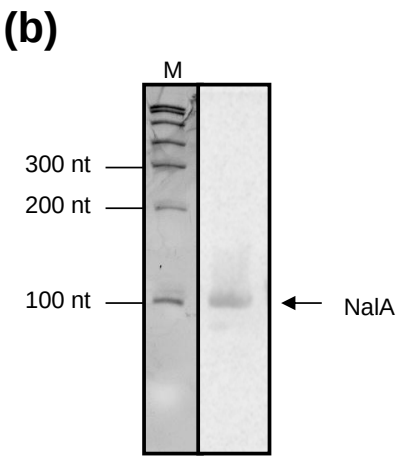
Fig. 4. Transcription of the PAO1 *nirB* gene occurs in the presence of nitrate and requires NalA. (a) RT-PCR analysis of *nirB* transcription in PAO1 four hours after shift to succinate minimal medium (BM2) and BM2 supplemented with nitrate. Upper panel: location of primers used for the RT-PCR analysis and expected length of the PCR products. Lower panel: In lanes denoted with a filled square and open square the primer pairs N55/I47 and N55/R64, respectively, were used. –RT, no reverse transcriptase added. (b) The β-galactosidase activity was determined in PAO1(pQF-*nalA-nirB*) and PAO1(pQF-Δ*nalA-nirB*) four hours after shift to BM2 medium containing nitrate. The deletion of *nalA* (position -5 to + 63) abolished *lacZ* expression. The experiment was performed in triplicate. Error bars, SD.

Fig. 5. NalA and NasT are required for nitrate assimilation in PAO1. Growth of strains PAO1 (diamonds), PAO1Δ*nalA* (a, squares) and PW4148 (b, squares) in succinate minimal medium supplemented with ammonium (NH_4^+, filled symbols) or nitrate (NO_3^-, open symbols). The experiment was done in duplicate; error bars, SD.

Fig. 6. Model for NalA and NasT/S dependent expression of the PAO1 nitrate assimilation operon. Upon nitrogen starvation, the glutamine:2-ketoglutarate ratio decreases, which leads to activation of the two-component system NtrBC and the σ^{54}-dependent *nalA* promoter. Under these conditions NalA is constitutively synthesized and NasT is likely sequestered by NasS. In the presence of nitrate, the NasS-mediated inhibition of NasT is alleviated, which results in antitermination within the NalA leader and in transcription of the PAO1 nitrate assimilation operon.

Figure 1





(c)

GCACC N_5 GGTGC GCACC N_5 GGTGC

CTGAAGGGCTGAGAGGCACCAGCTCGGTGCGATACGCCCCGGCACGGGGCAGAAAGACCGCGGGG

- 24 - 12

TTGGCA N_6 CTGCTT

AGGCCCCGCGTTATCCCTGCAAGGCCATGGAGATAGGCAACAGCGCAAG**TTGGCA**CGGCGC**CTGCTT**

A A **A T**

+ 1 *lacZ*

GGGTTCTCCCG**A**CAATCGACAACAAAGCGCCTGCCTCAACGACGAGGCGGCGTTTCCCGACCGAAC

GGGACCTGGACAAAGGCGTCCTGCTGGCAACAGCGGGACGCCTTTTTTGTTTGCGCCACCGC

Figure 2

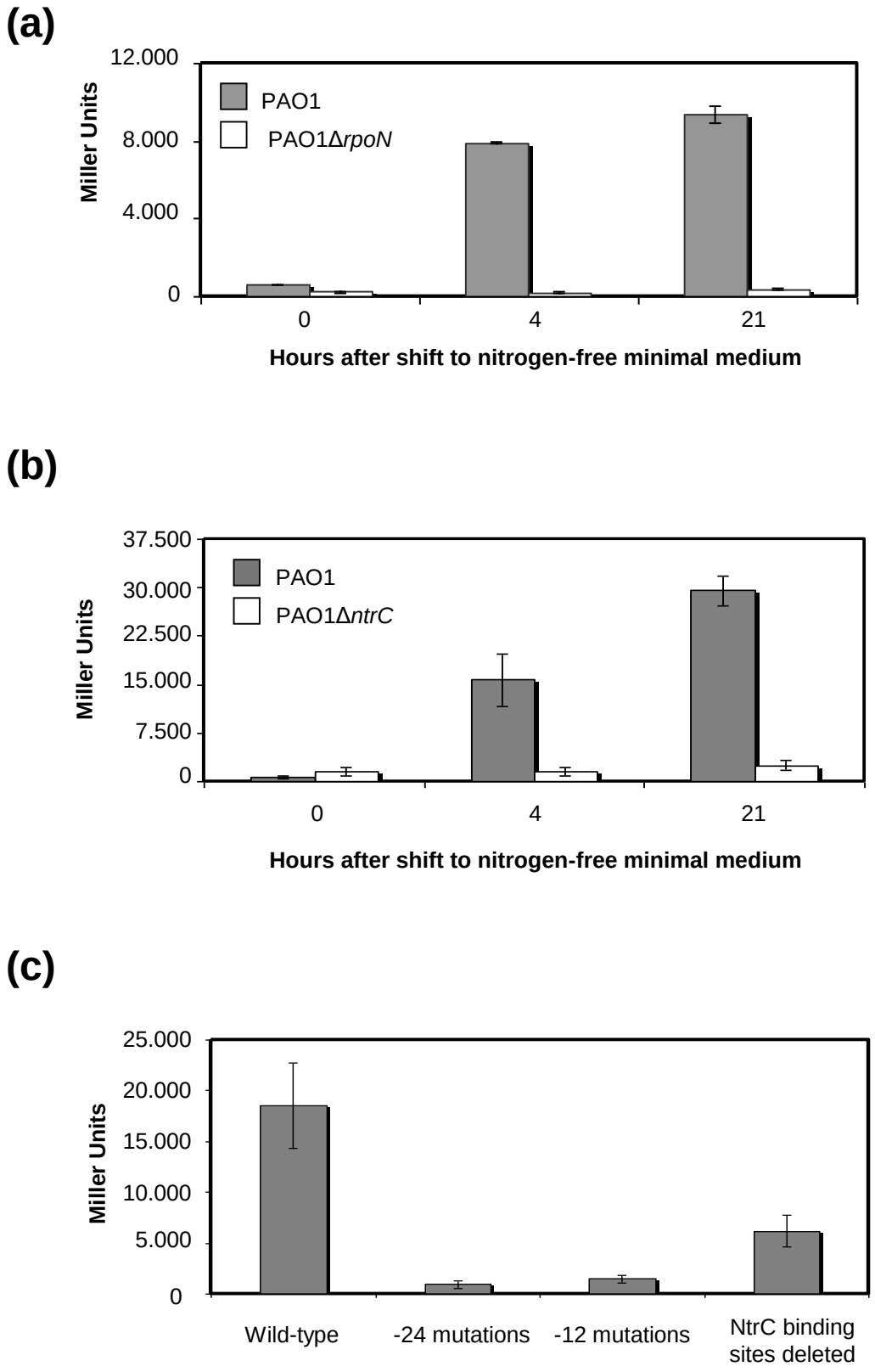

(a)
12.000
8.000
4.000
0
Miller Units
PAO1
PAO1Δrpon
0
4
21
Hours after shift to nitrogen-free minimal medium
(b)
37.500
30.000
22.500
15.000
7.500
0
Miller Units
PAO1
PAO1ΔntrC
0
4
21
Hours after shift to nitrogen-free minimal medium
(c)
25.000
20.000
15.000
10.000
5.000
0
Miller Units
Wild-type
-24 mutations
-12 mutations
NtrC binding sites deleted

Figure 3

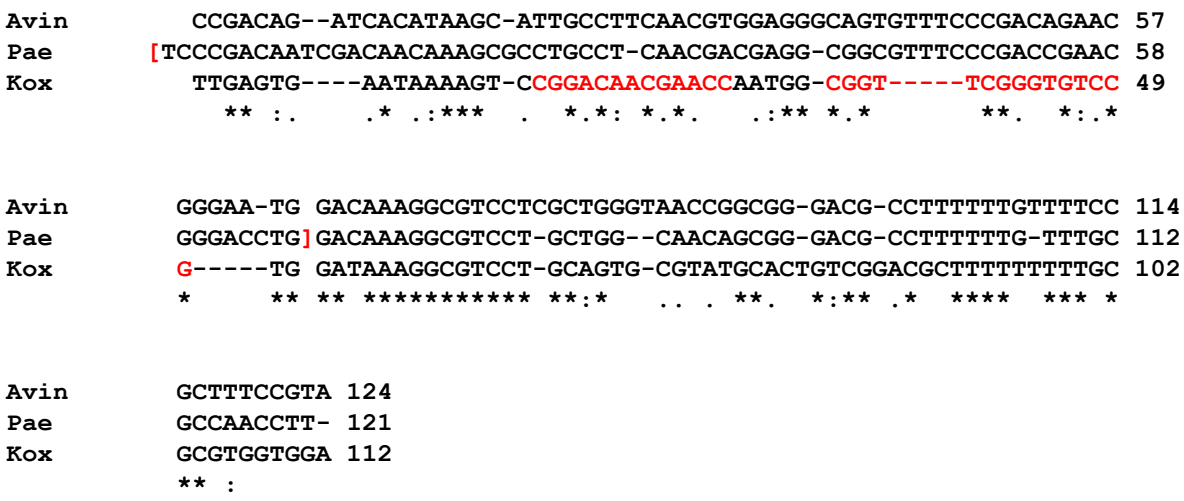
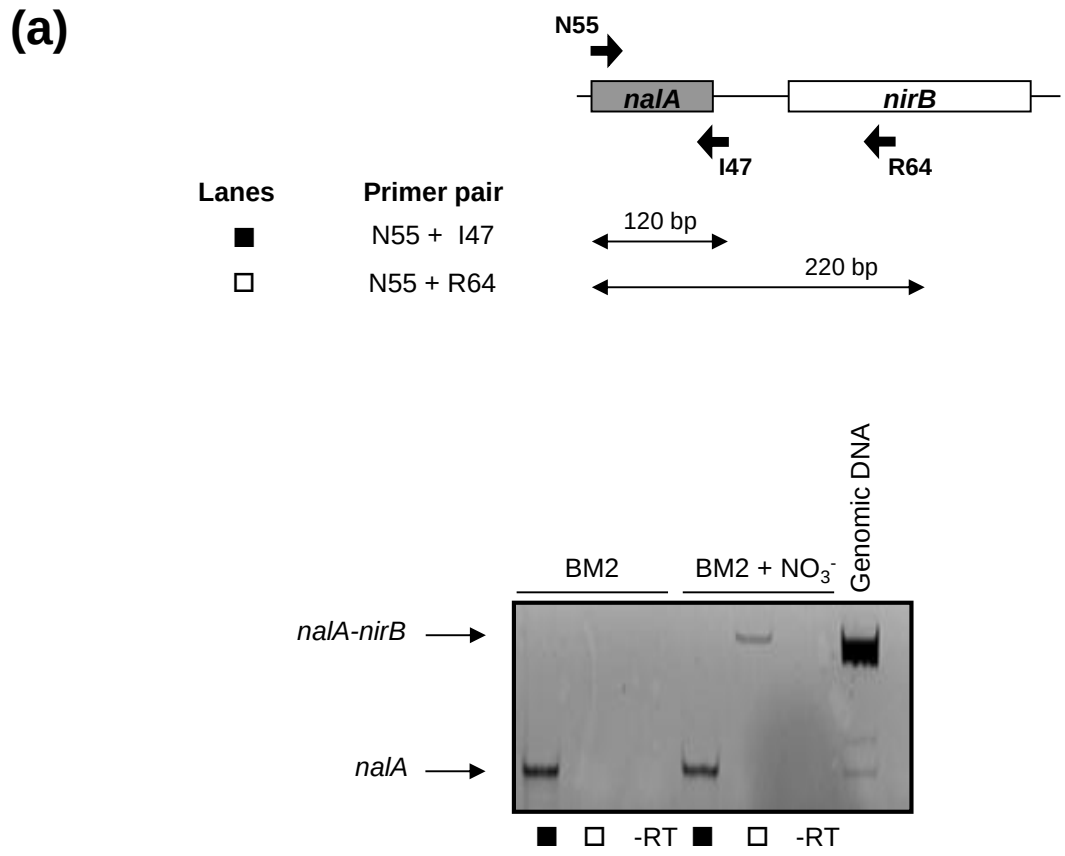

Avin CCGACAG--ATCACATAAGC-ATTGCCTTCAACGTGGAGGGCAGTGTTTCCCGACAGAAC 57
Pae [TCCCGACAATCGACAACAAAGCGCCTGCCT-CAACGACGAGG-CGGCGTTTCCCGACCGAAC 58
Kox TTGAGTG----AATAAAAGT-CCGGACAACGAACCAATGG-CGGT-----TCGGGTGTCC 49
** :. .* .:*** . *.*: *.*. .:** *.* **. *:.*
Avin GGGAA-TG GACAAAGGCGTCCTCGCTGGGTAACCGGCGG-GACG-CCTTTTTTGTTTTCC 114
Pae GGGACCTG]GACAAAGGCGTCCT-GCTGG--CAACAGCGG-GACG-CCTTTTTTG-TTTGC 112
Kox G-----TG GATAAAGGCGTCCT-GCAGTG-CGTATGCACTGTCGGACGCTTTTTTTTTGC 102
* ** ** ********** **:* .. . **. *:** .* **** *** *
Avin GCTTTCCGTA 124
Pae GCCAACCTT- 121
Kox GCGTGGTGGA 112
** :

Figure 4


(a)
N55
nalA
nirB
I47
R64
Lanes
Primer pair
N55 + I47
N55 + R64
120 bp
220 bp
BM2
BM2 + NO3-
Genomic DNA
nalA-nirB
nalA
-RT
-RT

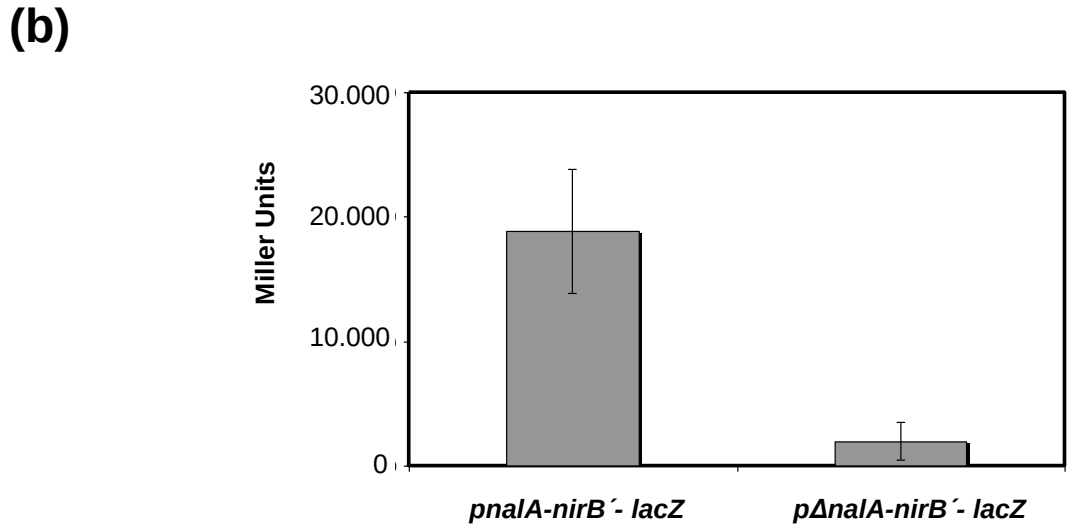

(b)
30.000
20.000
10.000
0
Miller Units
pnalA-nirB´- lacZ
pΔnalA-nirB´- lacZ

Figure 5

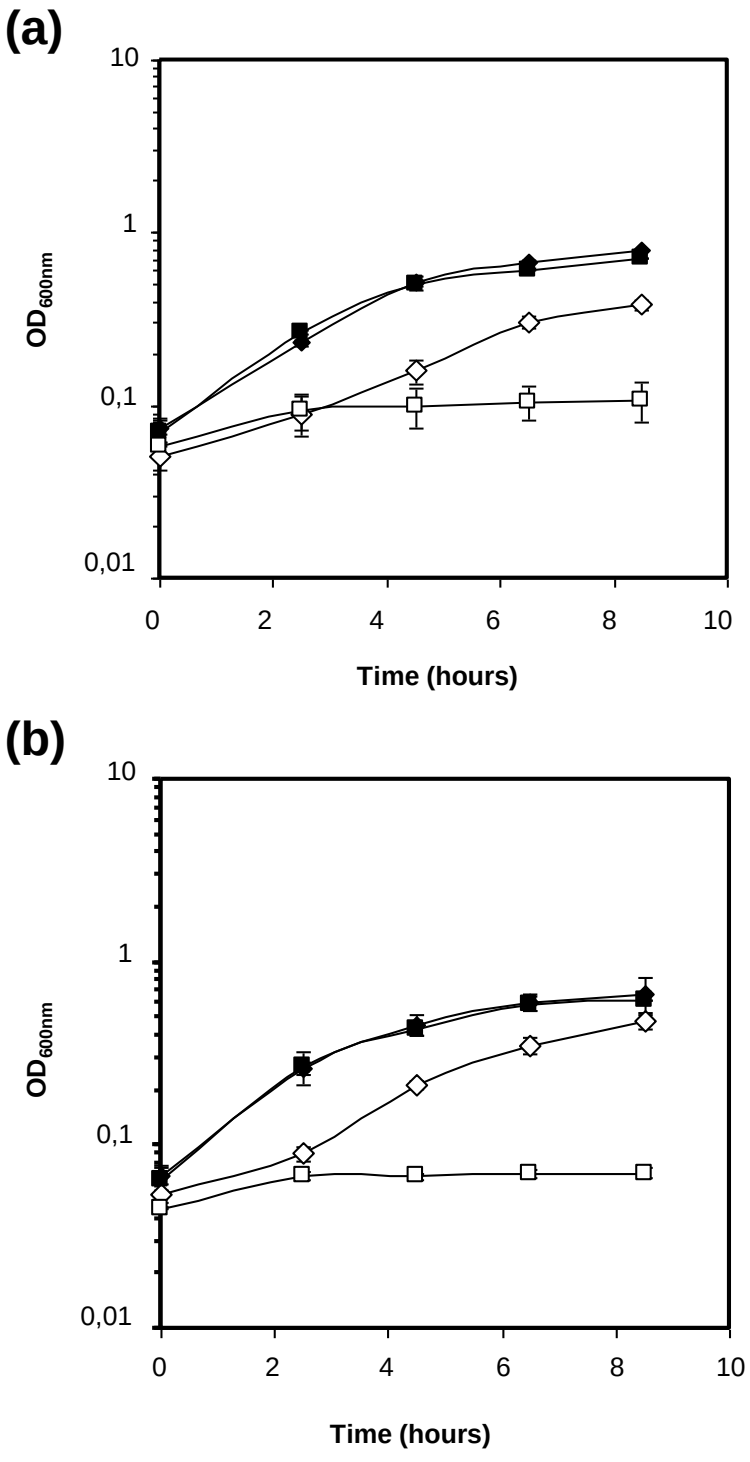
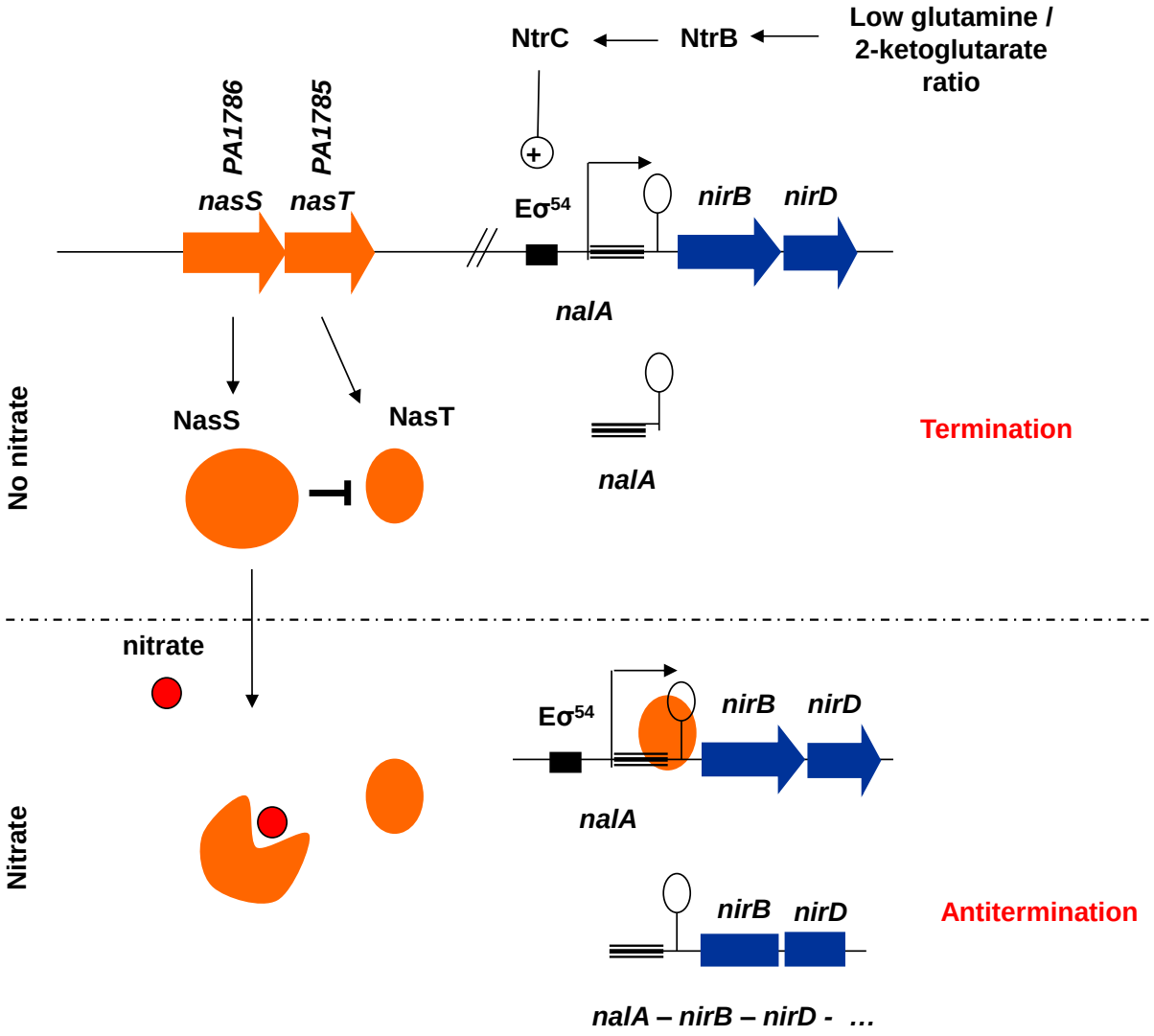

(a)
10
1
0,1
0,01
OD600nm
0
2
4
6
8
10
Time (hours)
(b)
10
1
0,1
0,01
OD600nm
0
2
4
6
8
10
Time (hours)

Figure 6


NtrC
NtrB
Low glutamine / 2-ketoglutarate ratio
PA1786
PA1785
nasS
nasT
Eσ54
nirB
nirD
nalA
No nitrate
NasS
NasT
Termination
nalA
nitrate
Eσ54
nirB
nirD
nalA
Nitrate
nirB
nirD
Antitermination
nalA – nirB – nirD - …

4.1.2. Supplementary Tables and Figures

Supplementary Table S1: DNA oligonucleotides used in this study.

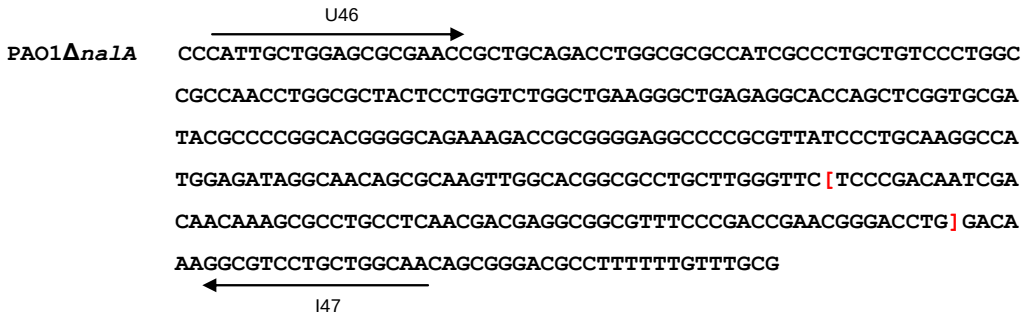
Name	Sequence (5´ to 3´)*	Restriction site	Binding region †
I26	CCCCACACTACCATCGGCGATGCGTCG		6039619 - 603964
I29	GC**TCTAGA**CCCCGCGTTATCCCTGCAAGGCCAT	*Xba*I	1928837 - 1928813
J29	A**GAATTC**AAACAAAAAAGGCGTCCCGCTGTTGC	*Eco*RI	1928656 - 1928681
U46	TTTT**GTCGAC**CATTGCTGGAGCGCGAAC	*Sal*I	1928991 - 1928974
V46	TTTT**GGATCC**CGTCGTTGAGGCAGGCGC	*Bam*HI	1928730 - 1928747
I47	TGCCAGCAGGACGCCT		1928679 - 1928694
L49	AAAA**CGATCG**TTGGCCAGTTGCATCGAC	*Pvu*I	1929453 - 1929436
M49	AAAA**GAATTC**GAACCCAAGCAGGCGCCGTG	*Eco*RI	1928699 - 1928680
N49	AAAA**GAATTC**GACAAAGGCGTCCTGCTGGC	*Eco*RI	1928768 - 1928787
O49	AAAA**CCATGG**GAACTGCACGGCACAGACCC	*Nco*I	1927949 - 1927968
M50	AGGTCCCGTTCGGTCGGGAAACGCCGCCTC		1928701 - 1928730
N55	ACAACAAAGCGCCTGCCTCAACG		1928762 - 1928733
R64	AAAA**GGATCC**CCGTTGCCGACCATCACCAGC	*Bam*HI	1928592 - 1928612
M57	GCAAGTTGGCACGGCGCCTTATTGGG		1928796 - 1928771
O63	GATAGGCAACAGCGCAAGTTAACACGGC		1928809 - 1928782
Q64	AACAAAGCGCCTGCCTCAACG		1928753 - 1928733
L80	CTCTCAGCCCTTCAGCC		1928891 - 1928907
A81	CAGAAAGACCGCGGGGA		1928856 - 1928840

* Restriction sites are highlighted in bold and the mutations introduced in the σ^{54}-dependent *nalA* promoter are underlined.

† The numbers indicate the complementary sequences on the PAO1 chromosome according to the *Pseudomonas* genome database http://www.pseudomonas.com.

Supplementary Figure S1

(a)



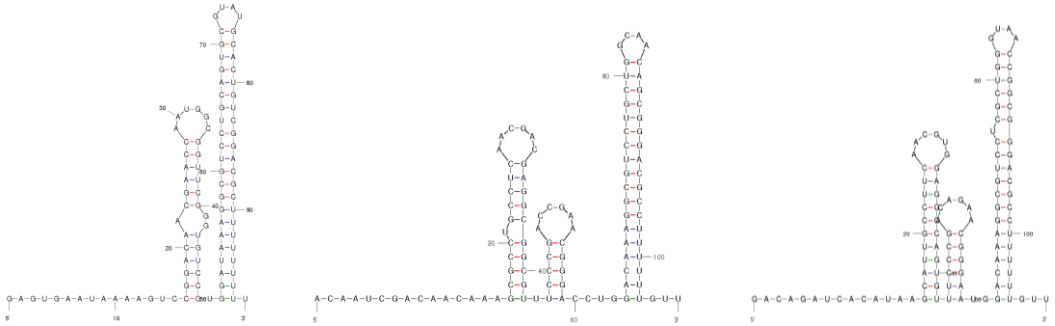
(b)

```
pQFnalA              TCGCCCTGCTGTCCCTGGCCGCCAACCTGGCGCTACTCCTGGTCTGGCTGAAGGGCTGAG 60
pQF12mutnalA         --GCCCTGCTGTCCCTGGCCGCCAACCTGGCGCTACTCCTGGTCTGGCTGAAGGGCTGAG 58
pQF24mutnalA         ----CCTGCTGTCCCTGGCCGCCAACCTGGCGCTACTCCTGGTCTGGCTGAAGGGCTGAG 56
pQFNtrCBSnalA        -CGCCCTGCTGTCCCTGGCCGCCAACCTGGCGCTACTCCTGGTCTGGCTGAAGGGCTGAG 59
                         ************************************************************

pQFnalA              AGGCACCAGCTCGGTGCGATACGCCCCGGCACGGGGCAGAAAGACCGCGGGGAGGCCCCG 120
pQF12mutnalA         AGGCACCAGCTCGGTGCGATACGCCCCGGCACGGGGCAGAAAGACCGCGGGGAGGCCCCG 118
pQF24mutnalA         AGGCACCAGCTCGGTGCGATACGCCCCGGCACGGGGCAGAAAGACCGCGGGGAGGCCCCG 116
pQFNtrCBSnalA        AG----------------------------------CAGAAAGACCGCGGGGAGGCCCCG 85
                     **                                  ************************

pQFnalA              CGTTATCCCTGCAAGGCCATGGAGATAGGCAACAGCGCAAATTGGCACGGCGCCTGCTTG 180
pQF12mutnalA         CGTTATCCCTGCAAGGCCATGGAGATAGGCAACAGCGCAAATTGGCACGGCGCCTTATTG 178
pQF24mutnalA         CGTTATCCCTGCAAGGCCATGGAGATAGGCAACAGCGCAAATTAACACGGCGCCTGCTTG 176
pQFNtrCBSnalA        CGTTATCCCTGCAAGGCCATGGAGATAGGCAACAGCGCAAATTGGCACGGCGCCTGCTTG 145
                     ****************************************  **********  ***

pQFnalA              GGTTCTCCCGACAATCGACAACAAAGCGCCTGCCTCAACGACGGGATCCCCGGGTACCCG 240
pQF12mutnalA         GGTTCTCCCGACAATCGACAACAAAGCGCCTGCCTCAACGACGGGATCCCCGGGTACCCG 238
pQF24mutnalA         GGTTCTCCCGACAATCGACAACAAAGCGCCTGCCTCAACGACGGGATCCCCGGGTACCCG 236
pQFNtrCBSnalA        GGTTCTCCCGACAATCGACAACAAAGCGCCTGCCTCAACGACGGGATCCCCGGGTACCCG 205
                     ************************************************************

pQFnalA              GGGATCTCTAGAAGCTTCTAGCTAGAGGGTATTAATAATGAAAGAGAATTCACTGGCCGT 300
pQF12mutnalA         GGGATCTCTAGAAGCTTCTAGCTAGAGGGTATTAATAATGAAAGAGAATTCACTGGCCGT 298
pQF24mutnalA         GGGATCTCTAGAAGCTTCTAGCTAGAGGGTATTAATAATGAAAGAGAATTCACTGGCCGT 296
pQFNtrCBSnalA        GGGATCTCTAGAAGCTTCTAGCTAGAGGGTATTAATAATGAAAGAGAATTCACTGGCCGT 265
                     ************************************************************
```

Supplementary Figure S1: (a) Deletion of the *nalA* sequence in PAO1Δ*nalA*. The deletion in the PAO1*nalA* strain (sequence within the red brackets) was confirmed by PCR and subsequent sequencing of the PCR fragment as described in Materials and Methods. (b) Depicted are the mutations introduced at either the -12 or -24 region of the σ^{54}-dependent *nalA* promoter, and the deletion of the two NtrC binding sites present in the *nalA* promoter region of plasmid pQFp*nalA*. The 34 bp deletion of the NtrC binding sites is indicated by a red bar. The GG to AA mutations at the -24 site and the GC to TA mutations at the -12 site of the σ^{54}-driven promoter are shown in red. The *Bam*HI restriction site used for construction of the transcriptional *lacZ* fusions is underlined. The Shine and Dalgarno sequence and the start codon of *lacZ* are shown in blue.

Supplementary Figure S2

(a)



K. oxytoca *P. aeruginosa* *A. vinelandii*

(b)

```
Avin        CCATGAAACCCCATAAGAGGGCGCACCGAACCAAG--ACAGTGCAAGCTG----GAACGA 54
Pae         ----GAGGCACCAGCTCGGTGCGATACGCCCCGGC--ACGGGGCA---------GAAAGA 45
Kox         --TAGATGCACAATCGATGGGCATTCTCTGCCTGCCGGCGGTGCACTTTTACGTGAAAAA 58
                ** * * *    * **       **      * * ***        ***  *

Avin        CTCCTTCAGGCCAGCCACCGGGAAATCCCT---------TGCAAATCAGTCATCCGTG-A 104
Pae         CCGC----GGGGAGGCCCCGCGTTATCCCTGCAAGGCCATGGAGAT-AGGCAACAGCGCA 100
Kox         CC------GGCCAACGTTGGGAAAAAGCCC----------GCCAGCGCGCCATTGCCA-A 101
            *       **  *      *    *  **          *       * **       *

                - 24        - 12
Avin        AACTGGCACAGCCCCTGCATCGAGAAAACCGACA---GATCACATAAGCATTGCCTTCAA 161
Pae         AGTTGGCACGGCGCCTGCTTGGGTTCTCCCGACAATCGACAACA-AAGCGCCTGCCTCAA 159
Kox         AGCTGGCATCCCGTTTGCATTACTTAT--TGA-----GTGAATAAAAGTCCGGAC---AA 151
            *  *****   *   *** *          **     *   * * ***      *   **

Avin        CGTGGAGGGCAGTGTTTCCCGACAGAACGGGAA-TGGACAAAGGCGTCCTCGC--TGGGT 218
Pae         CGACGAGG-CGGCGTTTCCCGACCGAACGGGACCTGGACAAAGGCGTCCT-GC--TGG-C 214
Kox         CGAACCAA-TGGCGGTTC--GGGTGTCCG-----TGGATAAAGGCGTCCT-GCAGTGCGT 202
            **         * * ***  *   *  **     **** *********** **  **

Avin        A---ACCGGCGGGACGCCTTTTTTGTTT---TCCGCTTTCCGTAACGGAGA-AGAGCG-A 270
Pae         A---AC-AGCGGGACGCCTTTTTTGTTTGCGCCAACCTTATGCA--GGAGCTACTCCGCA 268
Kox         ATGCACTGTCGG-ACGCTTTTTT--TTTG---------CGCGTGGTGGAGC----GGATA 246
            *   **   *** **** *****  ***            *    ****         *

Avin        TG 272
Pae         TG 270
Kox         TG 248
            **
```

Supplementary Figure S2: (a) Secondary structures of the PAO1 NalA RNA and its analogs in *K. oxytoca* and *A. vinelandii*, as predicted by the MFold algorithm[1]. (b) Sequence of the *nasF*, *nasA* and *nirB* operon control regions and leader sequences in *K. oxytoca*[2] (Kox), *A. vinelandii*[3] (Avin) and PAO1[4] (Pae), respectively. The sequences were aligned using CLUSTAL 2.0.12 multiple sequence alignment analysis[5,6]. Convergent arrows indicate the reported NtrC binding sites in *K. oxytoca*[2] and in PAO1. The conserved -24 and -12 motifs of the σ^{54} promoters are underlined. The transcriptional start site of *K. oxytoca, A. vinelandii* and PAO1 leaders are underlined and in italics. The translational start codon ATG for *nasF* (*K. oxytoca*), *nasA* (*A. vinelandii*) and *nirB* (PAO1) is shown in blue.

[1] **Zuker, M. (2003).** Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31(13),** 3406-3415.

[2] **Chai, W. & Stewart, V. (1999).** RNA sequence requirements for NasR-mediated, nitrate-responsive transcription antitermination of the *Klebsiella oxytoca* M5a1 *nasF* operon leader. *J Mol Microbiol* **292**, 203-216.

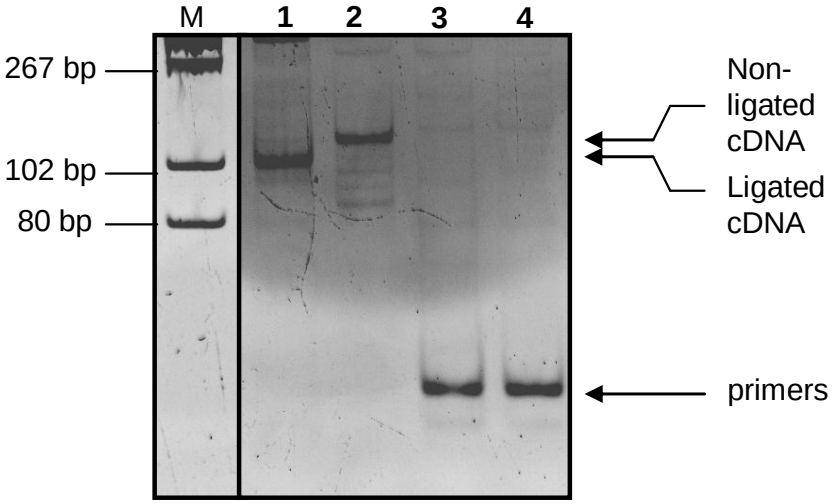
[3] **Setubal, J. C., dos Santos, P., Goldman, B. S., Ertesvåg, H., Espin, G., Rubio, L. M., Valla, S., Almeida, N. F., Balasubramanian, D. & other authors (2009).** Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. *J Bacteriol* **191(14)**, 4534-4545.

[4] **Stover, K. C., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J. & other authors (2000).** Complete genome sequence of *Pseudomonas aeruginosa* PAO1: an opportunistic pathogen. *Nature* **406**, 959-964.

[5] **Larkin, M. A. , Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007).** ClustalW and ClustalX version 2. *Bioinformatics* **23(21)**, 2947-2948.

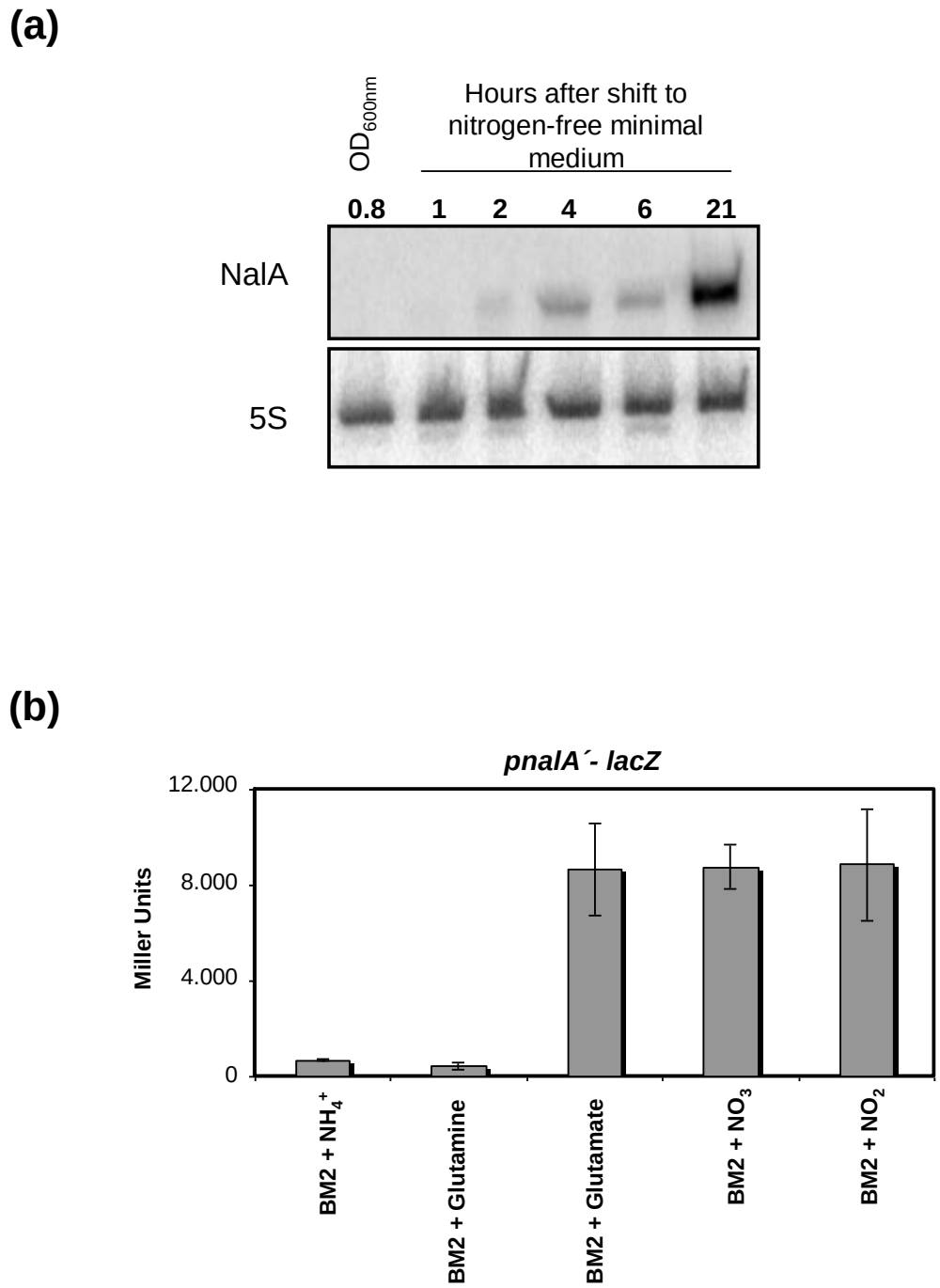
[6] **Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J. & Lopez, R. (2010).** A new bioinformatics analysis tools framework at EMBL-EBI. *Nucl Acids Res* **38**, 695-699.

Supplementary Figure S3



Supplementary Figure S3: Mapping of the 5´and 3´ends of NalA. Total RNA from PAO1 (pME*nalA*) grown in LB to stationary phase was TAP-treated and ligated, converted to cDNA and the 5´- 3´ junction was sequenced as described in Materials and Methods. Lane 1, RT-PCR of TAP-treated and ligated RNA. Lane 2, RT-PCR of non-TAP treated, non-ligated RNA. Lane 3, omission of RT. Lane 4, no RNA added. The size of the DNA markers (M) is indicated on the left (bp, base-pairs).

Supplementary Figure S4



Supplementary Figure S4: (a) NalA synthesis is induced upon nitrogen depletion. Total RNA from PAO1wt was purified using the hot phenol method[1]. The steady state levels of NalA in PAO1 grown in LB medium to an OD_{600} of 0.8 and at different times (hours) after shift to nitrogen free medium were determined by Northern-blot analysis using 10 µg total RNA. The RNA was denatured for 5 minutes at 65°C in loading buffer containing 50% formamide, separated on 8% polyacrylamide / 8M urea gels, and then transferred to nylon

membranes by semi-dry electroblotting. The RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized at 40°C with gene-specific ^{32}P-end labelled oligonucleotides (NalA, M50; 5S rRNA, I26; Supplementary Table S1) and the hybridisation signals were visualized using a PhosphoImager (Molecular Dynamics). (b) *nalA* promoter activity in response to different nitrogen sources. The p*nalA* driven transcription of the *lacZ* gene was monitored by measuring the β-galactosidase activity at an OD_{600} of 1.0 in PAO1(pQF*nalA*) grown in succinate minimal medium supplemented with the indicated nitrogen sources, at a concentration of 10 mM each. Only nitrite was added at a concentration of 5 mM. Error bars, SD.

[1] **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.

Supplementary Figure S5

(a)

```
Avin - NasT          MLRILLINDTPKKVGRLKSALVEAGFEVVDESGLTIDLPVRVEAVRPDVILIDTESPGRD 60
Pae - NasT (PA1785)  MLRILLIDDTPKKVGRLRAALLESGFEVVDESGLTIDLPARVEALRPDVILIDTESPGRD 60
                     *******:*********::**:*:***************.****:***************

Avin - NasT          VMEQVVLVSRDRPRPIVMFTDEHDPQVMRQAIQAGVSAYIVEGIQAQRLQPILDVAMARF 120
Pae - NasT (PA1785)  VMEQVCLVSRDQPRPIVMFTDEHDPGVMRRAIQSGVSAYIVEGIQASRLQPILDVAMARF 120
                     ***** *****:************* ***:***:************.*************

Avin - NasT          ESDQALRAQLQAREAQLAERKRVELAKGLLMKMKNCSEEEAYTLMRRQAMSRQQKLIQVA 180
Pae - NasT (PA1785)  ESDQALRAQLQAREAQLAERKRIELAKGLLMKMKHCGEEEAYTLMRRQAMSRQQRLIQVA 180
                     **********************:***********:*.*****************:*****

Avin - NasT          EQVIAMHDMLGS 192
Pae - NasT (PA1785)  EQIIAMHDMLGG 192
                     **:********.
```

(b)

```
Avin - NasS          MTDHHATFQSGCPGLGRGSDAPEKSSINLGFMPLTDSASLIVAATQGFAEPYGLTLNLKR 60
Pae - NasS (PA1786)  MTDRAIRSQAWAG----GSDAPEKSALDIGFMALTDSASLIVAATQGFAQPYGLTLNLRR 56
                     ***:    *: .     ********::::***.****************:********:*

Avin - NasS          QVSWSGLRDKLLSGELDAAQGLYGLIYSMQLGIGGAPATDMAVLMGLNQNGQSINLSTPL 120
Pae - NasS (PA1786)  QPSWATLRDKLLSGELDAAQCLYGLVYGVQLGLGGSAASEMAVLMGLCQNGQAINLSEPL 116
                     * **: ************** ****:*.:***:**:.*::******* ****:**** **

Avin - NasS          RQARVCSGETLVRHVRQSSANVTLAQTFPTGTHALWLYYWLASLGIHPLADVNTLVVPPP 180
Pae - NasS (PA1786)  KQAGVTSAEALLRHRRQNGARLTLAQTFPTGTHALWLNYWLASLGLHPLHDVHSVVVPPA 176
                     :** * *.*:*:** **..*.:*************** *******:*** **:::****.

Avin - NasS          QMVEHLRAGRSTVSAPESPAGP-RIDQGMGFTIATSQSIWPDHPEKSS-AAPCFAEQYPN 238
Pae - NasS (PA1786)  QMVGHLQAGRIDGFCAGGPWGALAVDQGQGFTIATSQAIWPDHPEKVLGTTRAFVDAYPN 236
                     *** **:***    .. .* *.  :*** ********:********   :: .*.: ***

Avin - NasS          TARALIMAVLNEP-LIDASEENKAGTAQLISANGYVAAPRQVIEPRFLGDYEDGNGMPGA 297
Pae - NasS (PA1786)  TARALVMAVLDASRFIEQNAENRLGTAQLISGRDYVDAPLGAIQPRFFGRYQDGLGNAWQ 296
                     *****:****: . :*: . **: *******...** **  .*:***:* *:** * .

Avin - NasS          DSMPCAFMGDGEVNLPYLSDGLWFMTQFRRWGLLREDPDYLAIATRVQQLELYRDAAGAL 357
Pae - NasS (PA1786)  DPHPLRFYADGEVNRPWLSDGMWFMTQFRRWGLLREDPDYLGIARRVQQTALYRDAATAL 356
                     *. *  * .***** *:****:*******************.** ****  ****** **

Avin - NasS          GMAHR-HGHAHATLLDGRRWDGSDPR------PMPAASTSTP---- 392
Pae - NasS (PA1786)  GLCLDGADMRRSTLIDGRTWDGSDPAGYARSFPIHALAETGQGIDL 402
                     *:.    .  ::**:*** ******       *: * : :
```

Supplementary Figure S5: (a) Similarity of the PAO1 PA1785 protein[1] and *A. vinelandii* NasT[2] revealed by CLUSTAL 2.0.12 multiple sequence alignment analysis[3,4]. Shown in red is the ANTAR motif (AmiR and NasR Transcriptional Antitermination Regulators) present in both proteins. (b) Similarity of the PAO1 PA1786 protein[1] and *A. vinelandii* NasS[2] protein revealed by CLUSTAL 2.0.12 multiple sequence alignment analysis[3,4].

[1] **Stover, K. C., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J. & other authors (2000).** Complete genome sequence of *Pseudomonas aeruginosa* PAO1: an opportunistic pathogen. *Nature* **406**, 959-964.

[2] **Setubal, J. C., dos Santos, P., Goldman, B. S., Ertesvåg, H., Espin, G., Rubio, L. M., Valla, S., Almeida, N. F., Balasubramanian, D. & other authors (2009).** Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. *J Bacteriol* **191(14)**, 4534-4545.

[3] **Larkin, M. A. , Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007).** ClustalW and ClustalX version 2. *Bioinformatics* **23(21)**, 2947-2948.

[4] **Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J. & Lopez, R. (2010).** A new bioinformatics analysis tools framework at EMBL-EBI. *Nucl Acids Res* **38**, 695-699

4.2. The leader RNA NalA of the *P. aeruginosa* nitrate assimilation operon appears not to function in *trans*

Small non-coding RNAs (sRNAs) are known to be modulators of gene expression. The *in silico* predicted sRNA NalA has been characterized as the leader RNA of the *nirBD* operon, which is transcribed only in the presence of nitrate. As NalA accumulates in the cell upon nitrogen starvation, I addressed the question whether NalA can regulate the expression of genes in *trans*. To test the impact of NalA on gene expression, a comparative transcriptome analysis of a *nalA* expressing strain and a *nalA* deletion strain was performed. This study revealed a moderate increase in transcript levels of genes required for oxidative stress defense, and of the zinc-dependent gene locus PA5534 to PA5541. The mRNA levels of the *dksA2* gene, encoding a transcriptional regulator, were found to be increased upon ectopic *nalA* expression. However, follow-up studies revealed that NalA does not impact on *dksA2* transcription.

INTRODUCTION

Typically expressed under stress conditions, regulatory RNAs are key players in the modulation of a wide range of physiological responses in Bacteria (Waters and Storz, 2009; Göpel & Görke, 2012). Bacterial small RNAs (sRNAs) can modulate the translation and stability of their target mRNA through base-pairing mechanisms. The structure and the binding region on the target mRNA determines the effect of a given sRNA. Binding of a sRNA to the ribosome binding site of a target mRNA can prevent its translation, and ultimately favours its decay (Massé *et al.*, 2003; Morita *et al.*, 2005). For instance, the *Pseudomonas aeruginosa* sRNAs PrrF1 and PrrF2 have been suggested to inhibit translation of mRNAs encoding iron-storage proteins under conditions of iron limitation (Wilderman *et al.*, 2004; Oglesby *et al.*, 2008; Oglesby-Sherrouse & Vasil, 2010).

Other sRNAs are able to stimulate translation of their target mRNA by melting a translationally unfavorable secondary structure masking the ribosome binding site (McCullen *et al.*, 2010; Soper *et al.*, 2010). One example in *P. aeruginosa* is the PhrS RNA. Expressed under anaerobic conditions, PhrS modulates quorum sensing and pyocyanin production by stimulating translation of the transcriptional regulator *pqsR* in an indirect manner. PhrS binds upstream of the *pqsR* gene and activates translation of a short reading frame to which translation of *pqsR* is positively coupled (Sonnleitner *et al.*, 2011).

Furthermore, sRNAs can sequester translational regulators, therefore inhibiting their function (reviewed in Marzi & Romby, 2012). In this way, the *P. aeruginosa* RNAs RsmY and RsmZ stimulate translation of genes required for chronic infection through sequestration of the translational repressor protein, RsmA (Pessi *et al.*, 2001; Sonnleitner *et al.*, 2006; Sorger-Domenigg *et al.*, 2006). Analogously, the regulatory small RNA CrcZ expressed in the presence of less favoured carbon sources in *P. aeruginosa* (i.e. mannitol) binds to and titrates

the translational repressor Crc, thereby permitting de-repression of Crc target mRNAs such as the aliphatic amidase-encoding *amiE* (Sonnleitner *et al.*, 2009). Hence, CrcZ enables the utilization of alternative carbon sources, which are subject to carbon catabolite repression in the presence of preferred carbon sources such as succinate (Görke & Stülke, 2008; Rojo, 2010).

In contrast to *trans*-encoded RNAs, which are usually not fully complementary to their mRNA target, *cis*-acting antisense RNA (asRNAs) form perfect duplexes with their mRNA targets (Kawano *et al.*, 2005). Transcribed from the complementary strand of a target gene, asRNAs can regulate expression of their target mRNA at the transcriptional or translational level. An example for transcriptional regulation represents the *Shigella flexneri* virulence gene *icsA*, which is regulated by the asRNA RnaG. Transcribed from a convergent promoter within *icsA*, the expression of both genes is on one hand controlled by transcriptional interference, whereby activation of the stronger *rnaG* promoter directly inhibits transcription from the weaker *icsA* promoter. Furthermore, the 450 nt long RnaG asRNA affects termination of *icsA* by base-pairing with its 5'UTR. This interaction modifies the structure of the *icsA* mRNA within the 5'UTR by creating a transcriptional terminator stem-loop. This transcriptional attenuation results in the synthesis of a truncated *icsA* mRNA (Giangrossi *et al.*, 2010). As translational modulators, asRNAs can bind to and promote the degradation of their targets mRNA. For instance, the transposition rate of the *E. coli* insertion sequence IS*10* (part of the TN*10* transposon) is regulated by the asRNA RNA-OUT. This asRNA is transcribed from a convergent promoter within the transposase *tnp* gene, and is thus complementary to the 5'UTR of the *tnp* mRNA (RNA-IN; Case *et al.*, 1988). Base-pairing of RNA-OUT and RNA-IN at the ribosome binding site blocks translation of the *tnp* mRNA (Ma & Simons, 1990). Recent studies revealed that Hfq is required for RNA-OUT mediated translational repression of *tnp* (Ross *et al.*, 2010).

At variation, *cis*-regulatory RNA elements can control transcription or translation of downstream genes. In response to a specific stimulus, such as the presence of a ligand, *cis*-regulatory RNAs can adopt a transcriptionally active structure or can fold into a terminator hairpin as exemplified in the *P. aeruginosa ami* operon for utilization of amides (Drew & Lowe, 1989). The terminator of the *amiL* leader RNA keeps the operon transcriptionally silent in the absence of amides. Expression of the operon requires antitermination of the leader by the RNA-binding protein AmiR and of the sensor protein AmiC in the presence of amides (Wilson & Drew, 1995; Drew & Haq, 2004). Another example for a *cis*-regulating RNA element is a thermosensor situated in the 5' UTR of the *Listeria monocytogenes prfA* mRNA. Translation of the *prfA* mRNA encoding a virulence regulator occurs at 37°C through melting of an intra-molecular secondary structure that masks the ribosome binding site. Thus, synthesis of PrfA can coincide with the infection of the host (Johansson *et al.*, 2002).

Other regulatory RNAs can function in *cis* as well as in *trans*. Following transcriptional termination, a *Listeria monocytogenes* riboswitch RNA accumulates in the presence of S-adenosylmethionine under metabolic stress. This transcript, SreA, can act in *trans* by base-pairing with target mRNAs. For instance, SreA inhibits translation of *prfA* by masking its ribosome binding site at 37°C, which is normally exposed by melting of the thermosensor structure (Loh *et al.*, 2009).

A recent study (Sayed *et al.*, 2011) in the Gram-positive human pathogen *Staphylococcus aureus* provided an example for a multifunctional RNA. The regulatory RNA SprA1 possesses an ORF encoding a 31 amino-acid cytolytic peptide. The translation of the peptide is under control of the asRNA $SpA1_{AS}$ transcribed from a convergent promoter located downstream of the *spA1* terminator. Despite sharing perfect complementarity with the SpA1 3'UTR, $SpA1_{AS}$ interaction with SpA1 occurs at the SprA1 ribosome binding site for the cy-

tolytic peptide. Consequently, the *cis*-antisense RNA $SprA1_{AS}$ actually functions in *trans* in repressing translation of the SprA1-encoded peptide.

The *P. aeruginosa* NalA (Nitrate/nitrite assimilatory leader RNA A) leader RNA was previously shown to control expression of the downstream genes *nirBD* through transcriptional termination in the absence of nitrate. Transcriptome analyses were performed to address the question whether the NalA RNA can additionally function as a trans-regulatory RNA during nitrogen stress. Despite a decrease in the RNA levels of genes coding for pilin (*flp*) and of the *fimUpilVWXY1*operon for production of type IV fimbriae in the presence of NalA, NalA did not impact on either motility or on biofilm formation. Similarly, the ~four-fold induction of the *dksA2* gene observed in the presence of NalA could not be attributed to a direct effect on *dksA2* transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani medium (Miller, 1972) supplemented with appropriate antibiotics. Antibiotics were added to a final concentration of 50 µg gentamycin ml^{-1}, 400 µg kanamycin ml^{-1} and 125 µg tetracycline ml^{-1}. Minimal salt medium (40 mM K_2HPO_4, 22 mM KH_2PO_4, 0,5 mM $MgSO_4$, 100 µM $Fe_2(SO_4)_3$, pH 7.0) was used for gene expression analysis. Sodium succinate (20 mM) was added as a carbon source, while sodium glutamate was added as a nitrogen source to a final concentration of 10 mM.

Construction of a vector for inducible NalA synthesis. The plasmid pJT*nalA* used for over-production of NalA under control of a toluic acid inducible promoter (*Pm* promoter) was constructed as follows. The *nalA* DNA sequence (bp +1 to + 222 with regard to the *nalA* transcriptional start site; see 4.1) was amplified by PCR using primers N55 (which includes the *Pm* promoter sequence) and O55, containing an *Xba*I and *Kpn*I restriction site, respectively (Table 2). The PCR fragment was then cloned into the corresponding sites of plasmid pJT19 (Table 1), resulting in pJT*nalA*.

RNA preparation and Northern-blotting. To determine the NalA abundance in PAO1, total RNA was purified using the hot phenol method (Lin-Chao and Bremer, 1986) from cells grown in minimal medium supplemented with glutamate to an OD_{600} of 0,5, 1 or 2. To verify induction of *nalA* in the PAO1Δ*nalA* strain, total RNA was purified from PAO1Δ*nalA* harbouring either plasmid pJT*nalA* or the parental vector pJT19. After induction of *nalA* with 2 mM toluic acid, the steady state levels of NalA were determined by Northern-blot analyses using 10 µg total RNA. The RNA was denatured for 5 minutes at 65°C in loading buffer containing 50% formamide, separated on 8% polyacrylamide / 8M urea gels, and then transferred

to nylon membranes (Amersham) by semi-dry electro-blotting. The RNA was cross-linked to the membrane by exposition to UV light. The membranes were hybridized at 40°C with gene-specific ^{32}P-end labelled oligonucleotides (NalA: M50; 5S rRNA: I26; Table 2) and the hybridisation signals were visualized using a PhosphoImager (Molecular Dynamics).

Expression profiling experiment. Strains PAO1Δ*nalA* (pJT19) and PAO1Δ*nalA* (pJT*nalA*) were grown in minimal medium supplemented with 20 mM succinate and 0,2% glutamate to an OD_{600} of 1,8. NalA synthesis was induced by addition of 2 mM toluic acid, and 10 ml of the culture was withdrawn 20 minutes post induction. The cell pellets were mixed with RNA Protect Bacteria reagent (Qiagen) and processed as recommended by the manufacturer. Total RNA was purified as previously described (Lin-Chao and Bremer, 1986). All experiments were performed in duplicate. For each experiment, 12 µg of RNA was sent to the Centre Intégratif de Génomique (Université de Lausanne), where the cDNA synthesis, cDNA fragmentation, 3′-end labelling, hybridization to GeneChip arrays (Affymetrix) and statistical analyses were performed. The fold change was considered to be significant, when the levels of individual genes differed at least 1,5-fold between PAO1Δ*nalA* (pJT19) and PAO1Δ*nalA* (pJT*nalA*).

Motility assays. Swimming of strains PAO1 and PAO1Δ*nalA* was tested by spotting a single colony of each tested strain on M9 minimal medium plates containing 0.3% agar, 20 mM succinate and 0,05% glutamate (w/v), and incubated overnight at 37 °C. For the twitching motility assay a bacterial colony of each tested strain (PAO1or PAO1Δ*nalA*) was stab inoculated on the bottom of a petri dish containing 10 ml of LB-agar and incubated overnight at 37°C. The swarming motility was determined by spotting a single clone of each tested strain (PAO1 or PAO1Δ*nalA*) on M9 minimal medium plates containing 0,5% agar, 20 mM succinate and 0,05% (w/v) glutamate. The plates were incubated overnight at 37 °C.

Static biofilm assays. The static biofilm assay was performed in a 96-well polystyrene microtiter plate. Overnight cultures of strains PAO1or PAO1Δ*nalA* grown in minimal medium supplemented with 20 mM succinate and 0,2% (v/v) glutamate were diluted to an OD_{600} of 0.5, and 200 µl of a 1:10 dilution was inoculated in a microtiter plate well. The attached Bacteria 24 hours post-inoculation were stained with 100 µl of 1% crystal violet for 15 min and washed twice with water. The wells were then rinsed with 400 µl 95% ethanol. Subsequently, 600 µl of water was added and the A_{570} was measured. All quantification assays were performed in triplicate.

Construction of plasmid pMEp*dksA2-lacZ* and β-galactosidase assays. For construction of the transcriptional p*dksA2-lacZ* fusion, the region comprising bp -109 to + 31 with regard to the start codon of the *dksA2* gene, was amplified by PCR using PAO1 genomic DNA together with forward primer D71, containing a *EcoR*I site, and reverse primer E71, containing a *Pst*I site (Table 2). The PCR product was cloned into the corresponding sites of plasmid pME6016, resulting in pMEp*dksA2-lacZ*. To monitor the influence of NalA expression on *dksA2* transcription, the PAO1Δ*nalA* strain harbouring pMEp*dksA2-lacZ* together with pJT*nalA* or the parental plasmid pJT19 were grown in LB medium under aeration at 37°C. At an OD_{600} of ~1.5, NalA synthesis was induced by addition of 2 mM toluic acid and one millilitre of culture was withdrawn at different times after induction. The β-galactosidase activity was determined as previously described (Miller, 1972). All experiments were done in triplicate.

RESULTS AND DISCUSSION

Induction of NalA alters the steady-state levels of several transcripts

The NalA RNA represents the leader RNA of the *nirBD*-PA1786-*cobA* operon required for nitrate assimilation in *Pseudomonas aeruginosa* (see 4.1). Expression of the operon is dependent on both nitrogen limitation and the presence of an inducer (nitrate or nitrite), and occurs through antitermination at the *nalA* rho-independent terminator. In the absence of nitrate, during nitrogen/amino-acid starvation, transcription is terminated at the *nalA* terminator. As NalA accumulates under these conditions (see 4.1.2, Figure S4A), we addressed the question whether NalA can as well function as a *trans*-acting RNA.

Using Northern-blot analysis, we first asked during which growth condition NalA accumulates. NalA was detected at an OD_{600} of 2 when PAO1 was grown in succinate minimal medium supplemented with glutamate (Figure 1A). These conditions were then used for a comparative transcriptome study with strains PAO1Δ*nalA* (pJT19) and PAO1Δ*nalA* (pJT*nalA*). The strains were grown in succinate minimal medium supplemented with glutamate, and *nalA* was induced at an OD_{600} of 1.8 by adding toluic acid to a final concentration of 2 mM. The steady-state levels of NalA were determined before and several times after induction (Figure 1B). In strain PAO1Δ*nalA* (pJT*nalA*), NalA was detectable 5 minutes after induction, reaching a plateau after 20 minutes. For the microarray analyses, total RNA was purified from samples withdrawn 20 minutes after addition of toluic acid. The short-term induction of NalA resulted in minor changes in the transcript levels of several genes. The genes whose expression was significantly altered by NalA represent less than 1%, whereby 71% of these transcripts showed an increase larger than 1.5-fold (Figure 1C).

NalA is not involved in motility and biofilm formation of PAO1

Swimming motility is strictly dependent on flagella, while twitching motility on solid, smooth surfaces (metal or plastic) is mediated by pili. On the other hand, swarming motility requires both appendages for translocation on semi-solid surfaces. As NalA expression had a slightly negative effect on the abundance of the *flp* transcript encoding a type IVb pilin, as well as on the *fimUpilVWXY1* operon for type IV pili biogenesis (Table 3), it was tested whether NalA affects bacterial motility. The overall spreading of the PAO1Δ*nalA* strain during swimming, swarming and twitching was indistinguishable from the PAO1 wild-type strain (Figure 2A).

The microcolonies forming the darker ring during twitching motility share homologies with the ones required for biofilm formation (O'Toole & Kolter, 1998). Moreover, the PilY1 protein was shown to control cell density by scanning cell-cell and cell-matrix contacts, and *pilY1* null mutants of PAO1 were shown to grow to higher cell densities when compared to parental strains (Tammy Bohn *et al.*, 2009). Consequently, the impact of NalA on biofilm formation was assessed (Figure 2B). It should be noted that the plate static biofilm growth assay is typically done in glucose- and casaminoacid-supplemented minimal medium (O'Toole & Kolter, 1998). Here, BM2 minimal medium supplemented with 0,05% glutamate as nitrogen source and either glucose or succinate as carbon source was used, as the induction of NalA is dependent on nitrogen limitation. The overall attached cells, determined by cristalviolet staining, did not differ between the PAO1 strain and the PAO1Δ*nalA* mutant strain, either 24 or 48 hours after initial inoculation. Consequently, the slightly reduced transcript levels of the *flp* gene and the *fimUpilVWXY1* operon upon *nalA* expression are not apparent in terms of motility or biofilm formation.

NalA does not affect *dksA2* expression at the transcriptional level

Microarray analyses identified the *dksA2* gene as the most up-regulated gene upon *nalA* induction (Table 3 and Figure 3A). The *dksA2* gene is a paralogue of the *dksA* gene (Blaby-Haas *et al.*, 2010). The two transcriptional repressor proteins differ with regard to their zinc dependence. DksA is zinc-dependent, whereas DksA2 is not (Blaby-Haas *et al.*, 2010). During exponential phase of growth, the zinc-dependent transcriptional repressor Zur binds to the *dksA2* promoter and prevents transcription. Conversely, de-repression of *dksA2* occurs in stationary phase, when a reduced availability for zinc impedes Zur activity (Blaby-Haas *et al.*, 2010).

Triggered by amino-acid or nutrient starvation, the stringent response in Pseudomonads is characterized by a rapid increase in the levels of (p)ppGpp (Greenway & England, 1999). In synergy with ppGpp, DksA favours the expression of amino-acid biosynthesis operons (Jude *et al.*, 2003), inhibits the quorum sensing circuitry *via* translational inhibition of *rhlI* (Branny *et al.*, 2001; Jude *et al.*, 2003) and reduces transcription of ribosomal protein genes (Perron *et al.*, 2005). DksA2 was also reported to inhibit transcription from the stringent *rnnB* P1 promoter, comparably to its zinc-dependent paralogue DksA (Blaby-Haas *et al.*, 2010). Moreover, DksA is involved in transcription from promoters recognized by alternative sigma factors, particularly that of σ^{54} (Bernardo *et al.*, 2006; reviewed in Potrykus and Cashel, 2008). The consequent increase in transcription from σ^{54}-dependent promoters due to DksA2 could therefore result in stimulation of *nalA* transcription as a positive feedback mechanism.

To verify the microarray data, we tested whether NalA induces transcription of *dksA2*. The promoter region of *dksA2* was fused to the reporter gene *lacZ*, and transcription from the promoter was monitored by measuring the β-galactosidase activity. However, in contrast to the microarray data, the induction of *nalA* did not result in an increase in *dksA2* transcription

in vivo, as no increase in the β-galactosidase activity was observed following toluic-acid-dependent *nalA* induction (Figure 3B).

Evidence was found for ppGpp and DksA involvement in σ^S translation in both, *Salmonella tiphimurium* (Webb *et al.*, 1999) and *E. coli* (Brown *et al.*, 2002). The increase in the transcript levels of some genes pertaining to the oxidative stress defense (Table 3), such as *lsfA* (Goldovà *et al.*, 2011), the operon PA1168-*loxA* (Vance *et al.*, 2004) and PA0284 (a homolog of *Pseudomonas corrugata* 28 *oscA* gene for sulphur metabolism and chromate resistance, Viti *et al.*, 2009) could derive from a DksA-mediated increase in σ^S activity, as seen for *E. coli* (McCann *et al.*, 1991). However, due to differences in lifestyle between *Pseudomonas* and *Escherichia coli*, the regulation and activity of σ^S differ as well (reviewed in Venturi, 2003). Indeed, the expression of many genes involved in stress defense is less dependent on σ^S in *P. aeruginosa* (Jørgensen *et al.*, 1999; Suh *et al.*, 1999). Consequently, the expression of these antioxidant-encoding genes in response to NalA induction may depend on other transcription factors such as OxyR, which is required for *lsfA* expression under oxidative stress in *P. aeruginosa* (Wei *et al.*, 2012).

A regulatory function for NalA?

The steady-state levels of an mRNA are determined by the rate of transcription and the rate of decay (Ross, 1995). Microarray-based methods for transcriptome profiling do not necessarily account for the stability of mRNAs. For instance, the decreasing abundance in *P. aeruginosa* DksA levels in late exponential and stationary phase of growth is linked to the reduced stability of its mRNA alone (Perron *et al.*, 2005).

The expression studies using the transcriptional *dksA2-lacZ* fusion construct could suggest that the possible stimulatory effect of NalA on *dksA2* occurs at a post-transcriptional

level. Thus, a translational p*dksA2*::*lacZ* fusion could be used to study possible regulatory effects of NalA on *dksA2* translation.

Finally, the results obtained with the p*dksA2-lacZ* fusion argue against a direct action of NalA on the transcriptional repressor Zur. Although NalA induction did not affect the steady-state levels of *zur-znuABC* genes (data not shown), which represent the most stringently controlled operon for zinc uptake (Patzer & Hantke, 2000; Shin *et al.*, 2011), another reason for the induction of the PA5534-41 locus could derive from deregulation of Zur activity, possibly through a reduction of the intracellular zinc levels.

REFERENCES

Case, C. C., Roels, S. M., González, J. E., Simons, E. L. & Simons, R. W. (1988). Analysis of the promoters and transcripts involved in IS*10* anti-sense RNA control. *Gene* **72(1-2)**, 219-236.

Drew, R. & Haq, M. (2004). Lessons from the *ami* operon. *Pseudomonas*: Virulence and Gene Regulation, ed Ramos JL (Kluwer Academic/Plenum, New York, NY), Vol 2, pp 425- 449.

Drew, R. & Lowe, N. (1989). Positive control of *Pseudomonas aeruginosa* amidase synthesis is mediated by a transcription anti-termination mechanism. *J Gen Microbiol* **135**, 817-823.

Bernardo, L. M. D., Johansson, L. U. M., Solera, D., Skärfstad, E. & Shingler, V. (2006). The guanosine tetraphosphate (ppGpp) alarmone, DksA and promoter affinity for RNA polymerase in regulation of σ^{54}-dependent transcription. *Mol Microbiol* **60(3)**, 749-764.

Blaby-Haas, C. E., Furman, R., Rodionov, D. A., Artsimovitch, I. & de Crécy-Lagard, V. (2010). Role of a Zn-independent DksA in Zn homeostasis and stringent response. *Mol Microbiol* **79(3)**, 700-715.

Branny, P., Pearson, J. P., Pesci, E. C., Kohler, T., Iglewski, B. H. & van Delden, C. (2001). Inhibition of quorum sensing by a *Pseudomonas aeruginosa dksA* homologue. *J Bacteriol* **183**, 1531-1539.

Brown, L., Gentry, D., Elliott, T. & Cashel, M. (2002). DksA affects ppGpp induction of RpoS at a translational level. *J Bacteriol* **184(6)**, 4455-4465.

Giangrossi, M., Prosseda, G., Tran, C. N., Brandi, A., Colonna, B. & Falconi, M. (2010). A novel antisense RNA regulates at transcriptional level the virulence gene *icsA* of *Shigella flexneri*. *Nucleic Acids Res* **38(10)**, 3362-3375.

Goldová, J., Ulrych, A., Hercík, K. & Branny, P. (2011). A eukaryotic-type signalling system of *Pseudomonas aeruginosa* contributes to oxidative stress resistance, intracellular survival and virulence. *BMC Genomics* **12**, 437.

Göpel, Y. & Görke, B. (2012). Rewiring two-component signal transduction with small RNAs. *Curr Opin Microbiol* **15(2)**, 132-139.

Görke, B. & Stülke, J. (2008). Carbon catabolite repression in Bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* **6**, 613-624.

Greenway, D. L. & England, R. R. (1999). ppGpp accumulation in *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* subjected to nutrient limitation and biocide exposure. *Lett Appl Microbiol* **29(5)**, 298-302.

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

Johansson, J., Mandin, R., Renzoni, A., Chiaruttini, C., Springer, M. & Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes. Cell* **110**, 551-561.

Jørgensen, F., Bally, M., Chapon-Herve, V., Michel, G., Lazdunski, A., Williams, P. & Stewart, G. S. (1999). RpoS dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* **145**, 835-844.

Jude, F., Kohler, T., Branny, P., Perron, K., Mayer, M. P., Comte, R. & van Delden, C. (2003). Posttranscriptional control of quorum-sensing-dependent virulence genes by DksA in *Pseudomonas aeruginosa*. *J Bacteriol* **185**, 3558-3566.

Kawano, M., Reynolds, A. A., Miranda-Rios, J. & Storz, G. (2005). Detection of 5'- and 3'-UTR-derived small RNAs and *cis*-encoded antisense RNAs in *Escherichia coli*. *Nucleic Acids Res* **33**, 1040-1050.

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.

Loh, E., Dussurget, O., Gripenland, J., Vaitkevicius, K., Tiensuu, T., Mandin, P., Repoila, F., Buchrieser, C., Cossart, P. & Johansson, J. (2009). A *trans*-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*. *Cell* **139(4)**, 770-779.

Ma, C. & Simons, R. W. (1990). The IS*10* antisense RNA blocks ribosome binding at the transposase translation initiation site. *EMBO J* **9(4)**, 1267-1274.

Marzi, S. & Romby, P. (2012). RNA mimicry, a decoy for regulatory proteins. *Mol Microbiol* **83(1)**, 1-6.

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

McCann, M. P., Kidwell, P. & Matin, A. (1991). The putative σ-factor KatF has a central role in development of starvation mediated general resistance in *Escherichia coli. J Bacteriol* **173**, 4188-4194.

McCullen, C. A., Benhammou, J. N., Majdalani, N. & Gottesman, S. (2010). Mechanism of positive regulation by DsrA and RprA small noncoding RNAs: pairing increases translation and protects *rpoS* mRNA from degradation. *J Bacteriol* **192(21)**, 5559-5571.

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

Morita, T., Maki, K. & Aiba, H. (2005). RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev* **19(18)**, 2176-2186.

Novichkov, P. S., Laikova, O. N., Novichkova, E. S., Gelfand, M. S., Arkin, A. P., Dubchak, I. & Rodionov, D. A. (2010). RegPrecise: a database of curated genomic inferences of transcriptional regulatory interactions in prokaryotes. *Nucleic Acids Res* **38(Database issue)**, D111-118**.**

Oglesby, A. G., Farrow, J. M. 3rd, Lee, J. H., Tomaras, A. P., Greenberg, E. P., Pesci, E. C. & Vasil, M. L. (2008). The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. *J Biol Chem* **283(23)**, 15558-15567.

Oglesby-Sherrouse, A. G. & Vasil, M. L. (2010). Characterization of a heme-regulated non-coding RNA encoded by the *prrF* locus of *Pseudomonas aeruginosa*. *PLoS One* **5(4)**, e9930.

O'Toole, G. A. & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**, 295-304.

Patzer, S. I. & Hantke, K. (2000). The zinc-responsive regulator Zur and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. *J Biol Chem* **275(32)**, 24321-24332.

Perron, K., Comte, R. & van Delden, C. (2005). DksA represses ribosomal gene transcription in *Pseudomonas aeruginosa* by interacting with RNA polymerase on ribosomal promoters. *Mol Microbiol* **56(4)**, 1087-1102.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Cámara, 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.

Potrykus, K. & Cashel, M. (2008). (p)ppGpp: still magical? *Annu Rev Microbiol* **62**, 35-51.

Rojo, F. (2010). Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and the interaction with the environment. *FEMS Microbiol Rev* **34**, 658-784.

Ross, J. (1995). mRNA stability in mammalian cells. *Microbiol Rev* **59**, 423-450.

Ross, J. A., Wardle, S. J. & Haniford, D. B. (2010). Tn*10*/IS*10* transposition is downregulated at the level of transposase expression by the RNA-binding protein Hfq. *Mol Microbiol* **78(3)**, 607-621.

Sayed, N., Jousselin, A. & Felden, B. (2011). A *cis*-antisense RNA acts in *trans* in *Staphylococcus aureus* to control translation of a human cytolytic peptide. *Nat Struct Mol Biol* **19(1)**, 105-112.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, 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.

Shin, J. H., Jung, H. J., An, Y. J., Cho, Y. B., Cha, S. S. & Roe, J. H. (2011). Graded expression of zinc-responsive genes through two regulatory zinc-binding sites in Zur. *Proc Natl Acad Sci USA* **108(12)**, 5045-5050.

Sonnleitner, E., Abdou, L. & Haas, D. (2009). Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **106**, 21866-21871.

Sonnleitner, E., Gonzalez, N., Sorger-Domenigg, T., Heeb, S., Richter, A. S., Backofen, R., Williams, P., Hüttenhofer, A., Haas, D. and other authors (2011). The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. *Mol Microbiol* **80(4)**, 868-885.

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(5)**, 1542-1558.

Soper, T., Mandin, P., Majdalani, N., Gottesman, S. & Woodson, S. A. (2010). Positive regulation by small RNAs and the role of Hfq. *Proc Natl Acad Sci USA* **107(21)**, 9602-9607.

Sorger-Domenigg, T., Sonnleitner, E., Kaberdin, V. R. & Bläsi, U. (2006). Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY. *Bioch Biophys Res Comm* **352**, 769-773.

Suh, S. J., Silo-Suh, L., Woods, D. E., Hassett, D. J., West, S. E. & Ohman, D. E. (1999). Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 3890-3897.

Tammy Bohn, Y. S., Brandes, G., Rakhimova, E., Horatzek, S., Salunkhe, P., Munder, A., van Barneveld, A., Jordan, D., Bredenbruch, F., Häußler, S. & other authors (2009). Multiple roles of *Pseudomonas aeruginosa* TBCF10839 PilY1 in motility, transport and infection. *Mol Microbiol* **71(3)**, 730-747.

Vance, R. E., Hong, S., Gronert, K., Serhan, C. N. & Mekalanos, J. J. (2004). The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase. *Proc Natl Acad Sci USA* **101(7)**, 2135-2139.

Venturi, V. (2003). Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? *Molecular Microbiology* **49(1)**, 1-9.

Viti, C., Decorosi, F., Mini, A., Tatti, E. & Giovannetti, L. (2009). Involvement of the *oscA* gene in the sulphur starvation response and in Cr(VI) resistance in *Pseudomonas corrugata* 28. *Microbiology* **155**, 95-105.

Waters, L. S. & Storz, G. (2009). Regulatory RNAs in Bacteria. *Cell* **136**, 615-628.

Wei, Q., Le Minh, P. N., Dötsch, A., Hildebrand, F., Panmanee, W., Elfarash, A., Schulz, S., Plaisance, S., Charlier, D, & other authors (2012). Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nucleic Acids Res* **40(10)**, 4320-4333.

Webb, C., Moreno, M., Wilmes-Riesenberg, M., Curtiss, R. 3rd & Foster, J. W. (1999). Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium*. *Mol Microbiol* **34(1)**, 112-123.

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 USA* **101(26)**, 9792-9797.

Wilson, S. A. & Drew, R. E. (1995). Transcriptional analysis of the amidase operon from *Pseudomonas aeruginosa*. *J Bacteriol* **177(11)**, 3052-3057.

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

Table 1: Bacterial strains and plasmids

Strain or Plasmid	Genotype/Relevant features	Reference or Origin
***P. aeruginosa* strains**		
PAO1	Wild-type	Holloway *et al.* (1979)
PAO1*ΔnalA*	*nalA* deletion strain	Refer to 4.1
***E. coli* strain**		
Top 10	F-, *mcrA*, D(*mrr-hsd*RMS-*mcr*BC),p80*lacZ*DM15 D*lacX*74, *deoR*, *recA1*, *araD*139D(*ara-leu*)7697, *galK*, *rpsL*(Str^R), *endA1*, *nupG*	Stratagene
Plasmids		
pJT19	Broad-host range expression vector (*Pm* promoter), Kan^R	Winther-Larsen *et al*. (2000)
pJT*nalA*	pJT19 carrying *nalA* (toluic acid inducible promoter *Pm*), Kan^R	This study
pME6016	Cloning vector for transcriptional *lacZ* fusions, Tc^R	Schnider-Keel *et al.* (2002)
pMEp*dksA2-lacZ*	pME6016 carrying a transcriptional *dksA2-lacZ* fusion (-109 to +31 bp with respect to the *lacZ* ATG start codon), Tc^R	This study

Table 2: DNA oligonucleotides used in this study

Name	Sequence (5´ to 3´end)*	Restriction site	Binding region †
J29	A**GAATTC**AAACAAAAAAGGCGTCC CGCTGTTGC	*Eco*RI	1928656 - 1928681
M50	AGGTCCCGTTCGGTCGGGAAACGC CGCCTC		1928701 - 1928730
N55	AAAA**TCTAGA**AAGGCCTACCCCTT AGGCTTTATGCAACAATCGACAACA AAGCGCCTGCCTCAACG	*Xba*I	1928762 - 1928733
O55	AAAA**GGTACC**AAGTCCGAGTTGAG-CTTGAGCAGTTCTTCC	*Kpn*I	1928541 - 1928570
D71	TTTT**GAATTC**TGGATCCCGGGAT-GACGTGG	*EcoRI*	6229971 - 6229952
E71	TTTT**CTGCAG**CGGGCTGGGCAAG-CAGTTCC	*PstI*	6229832 - 6229851

* Restriction sites are in bold and the promoter sequence for T7 polymerase (R5, U26) and the *Pm* promoter (N55), are underlined.

† The numbers indicate the complementary segments on the PAO1 chromosome according to the *Pseudomonas* genome database http://www.pseudomonas.com.

Table 3: Selected NalA-regulated genes

Identification [a]	Gene name	Fold change [b]
Unknown function		
PA3446		+ 1,98
PA3601		+1,85
PA3931		+1,95
PA4139		+1,87
PA4826		+2,01
Motility and attachment		
PA4306	*flp*	- 2,19
PA4551	*pilV*	- 1,54
PA4552	*pilW*	- 1,72
PA4553	*pilX*	- 1,5
PA4554	*pilY1*	- 1,57
Genes of the oxidative stress response		
PA0201		+ 1,94
PA0284	*oscA* [c]	+ 3,77
PA1168		+ 2,40
PA1169	*loxA*	+ 1,93
PA3450	*lsfA*	+ 3,43
Related to zinc availability		
PA5534		+2,27
PA5535		+ 2,42
PA5536	*dksA2*	+ 3,97
PA5538	*amiA*	+ 2,41
PA5540		+2,15
PA5541	*pyrQ*	+ 2,23

[a] DNA microarray analyses were carried out as described in Materials and Methods. The genes identified to be up- or down regulated in the PAO1Δ*nalA* (pJT*nalA*) strain were grouped according to their function.

[b] The fold-change represents the mean of the transcript levels in PAO1Δ*nalA* (pJT*nalA*) versus PAO1Δ*nalA* (pJT19) strains from four independent measurements. The table includes data for down regulated genes with a fold-change < -1,8 and up-regulated genes with a fold-change > 1,8 with the exception of the *fimUpilVWXY1* operon.

[c] PA0284 name was designated according to the orthologous gene *oscA* of *P. stuzeri.*

FIGURE LEGENDS

Figure 1. (A) NalA transcript abundance was assessed by Northern blot analysis of total RNA purified from PAO1 grown in succinate minimal medium supplemented with glutamate. (B) Toluic acid-dependent induction of NalA synthesis in *P. aeruginosa* PAO1Δ*nalA* carrying plasmid pJT*nalA*. PAO1Δ*nalA* harbouring the parental plasmid pJT19 was used as a control. Both strains were grown in minimal medium supplemented with succinate and glutamate at 37°C until they reached an OD_{600} of 1.8 (= time 0). Toluic acid was then added to the bacterial suspension. Samples were harvested at the indicated times post induction and total RNA from these samples was used for Northern-blot analysis to monitor the steady-state levels of NalA. (C) Functional classification of NalA-dependent genes according to the *Pseudomonas* Genome Project (http://www.pseudomonas.com). The values represent the number of genes affected by NalA in PAO1Δ*nalA* (pJT*nalA*) versus PAO1Δ*nalA* (pJT19) within the respective class.

Figure 2: Analysis of NalA effects on PAO1 motility, attachment and biofilm formation. (A) Swimming, swarming and twitching motility was tested for the indicated *P. aeruginosa* strains. (B) Quantification of crystal violet staining of 24 hours biofilms of PAO1 (black) and PAO1Δ*nalA* (white) strains grown in 96-wells microtiter plates in minimal medium supplemented with 0,2% glutamate in the presence of the indicated carbon source (final concentration 20 mM). Error bars, SD.

Figure 3: (A) Genetic organization of NalA-induced gene operons PA5534-*dksA2* and PA5539-*pyrC2*. For each transcript, the observed increase upon *nalA* induction is given un-

derneath. The putative Zur binding motif (from the http://regprecise.lbl.gov website, Novichkov *et al.*, 2010) is indicated by a red bar. (B) Transcription of the p*dksA2-lacZ* transcriptional fusion gene was monitored by measuring the β-galactosidase activity at the indicated time following addition of toluic acid to strains PAO1Δ*nalA* (pJT19) (filled symbols) and PAO1Δ*nalA* (pJT*nalA*) (open symbols) grown in LB medium. The experiment was performed in triplicate. Error bars, SD.

Figure 1

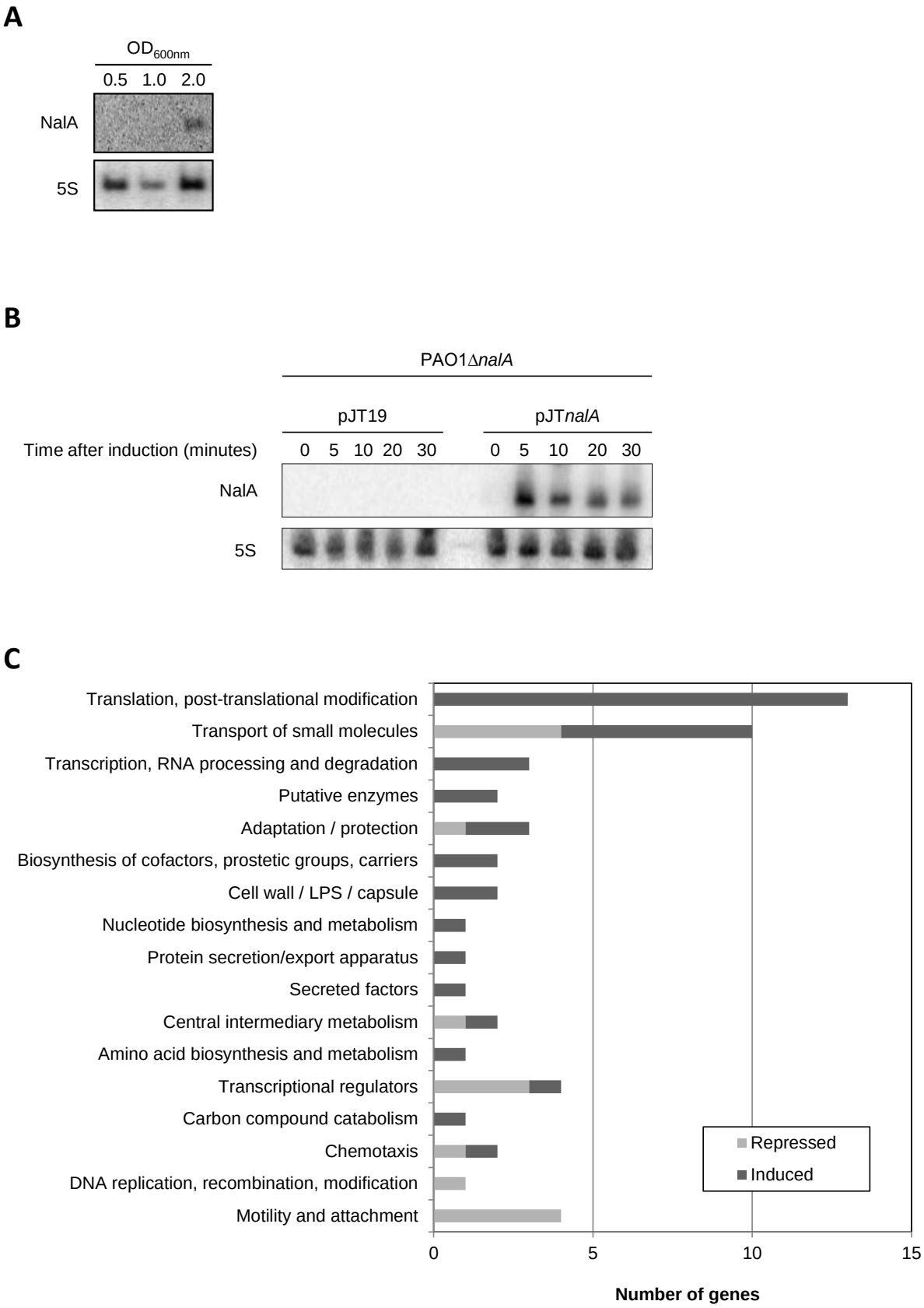
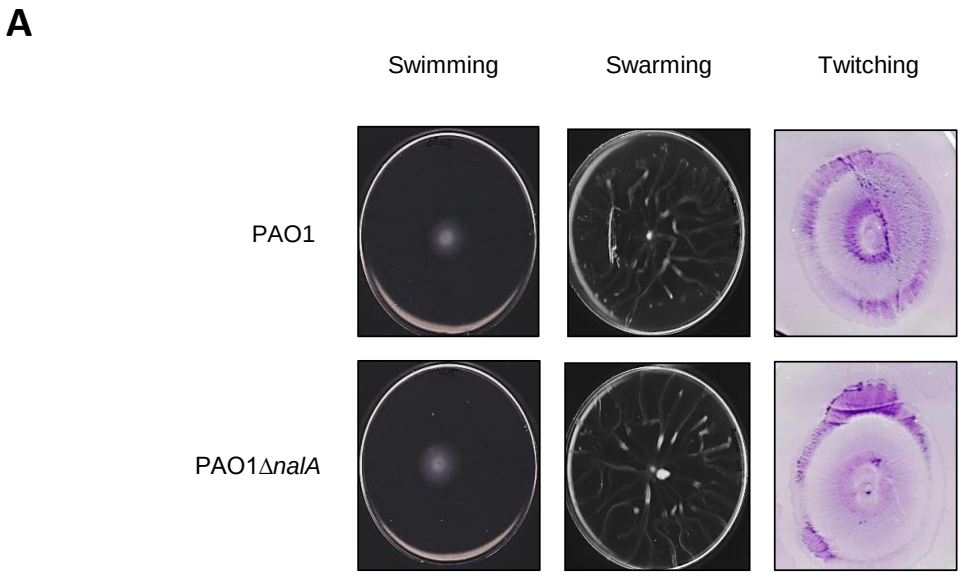

A
OD600nm
0.5 1.0 2.0
NalA
5S
B
PAO1ΔnalA
pJT19
pJTnalA
Time after induction (minutes)
0 5 10 20 30 0 5 10 20 30
NalA
5S
C
Translation, post-translational modification
Transport of small molecules
Transcription, RNA processing and degradation
Putative enzymes
Adaptation / protection
Biosynthesis of cofactors, prostetic groups, carriers
Cell wall / LPS / capsule
Nucleotide biosynthesis and metabolism
Protein secretion/export apparatus
Secreted factors
Central intermediary metabolism
Amino acid biosynthesis and metabolism
Transcriptional regulators
Carbon compound catabolism
Chemotaxis
DNA replication, recombination, modification
Motility and attachment
Repressed
Induced
0
5
10
15
Number of genes

Figure 2


A
Swimming
Swarming
Twitching
PAO1
PAO1ΔnalA

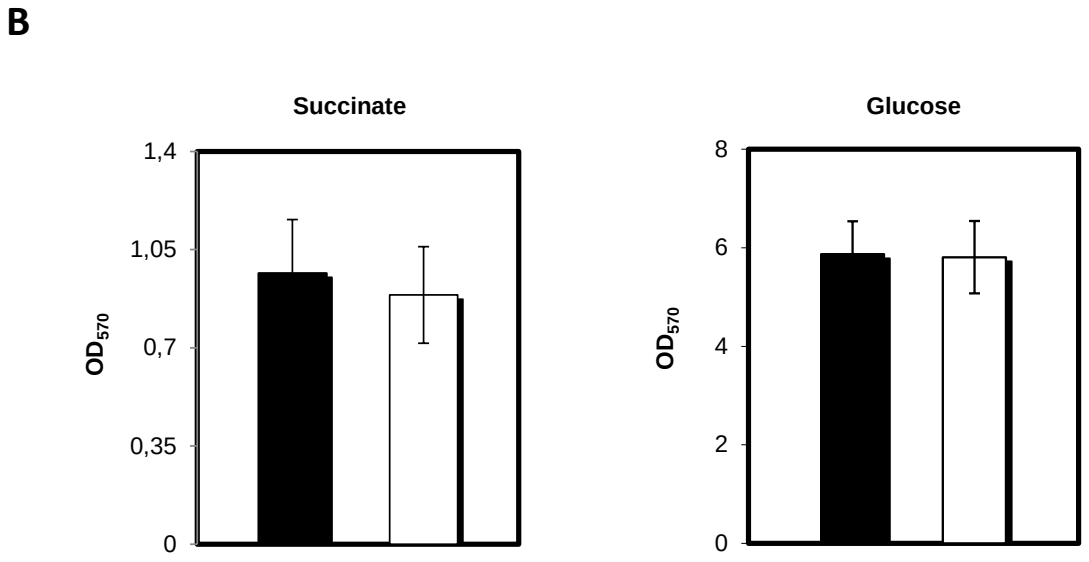
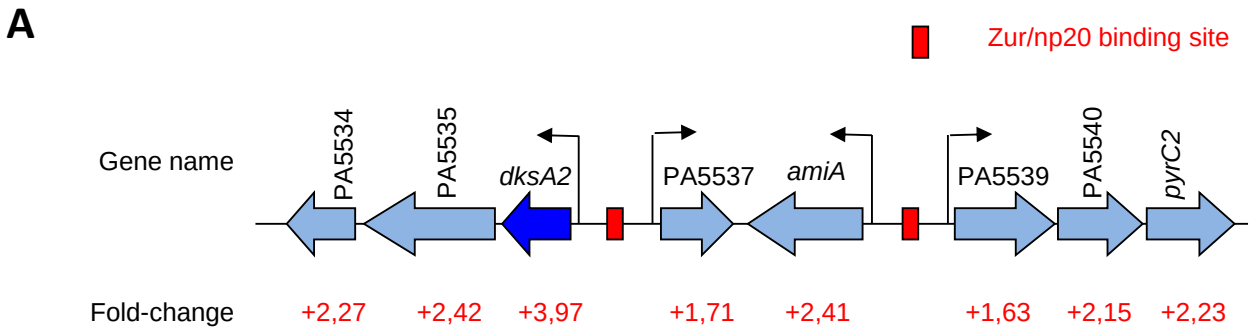
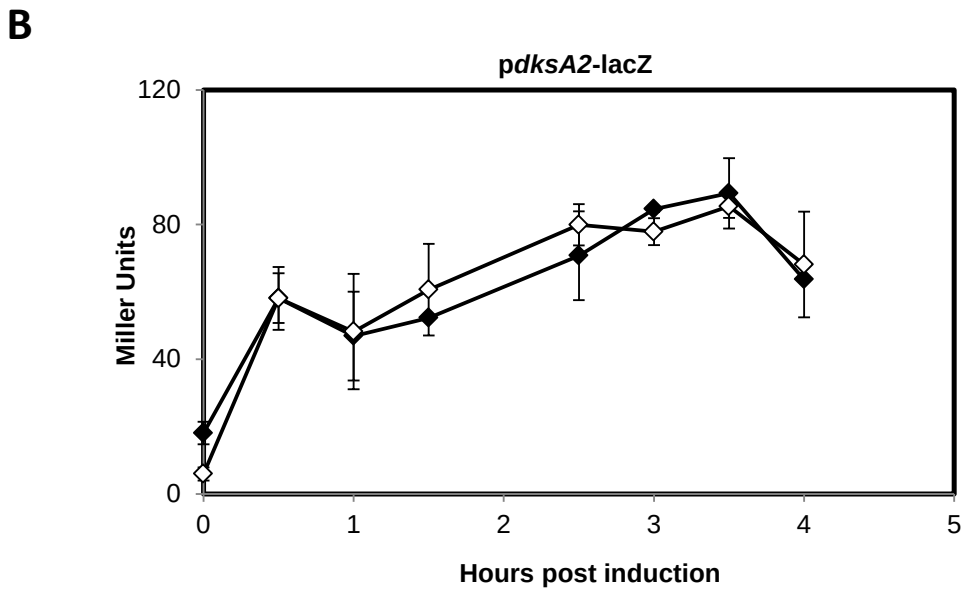

B
Succinate
Glucose
OD570
1,4
1,05
0,7
0,35
0
8
6
4
2
0

Figure 3


A
Zur/np20 binding site
Gene name
PA5534
PA5535
dksA2
PA5537
amiA
PA5539
PA5540
pyrC2
Fold-change
+2,27
+2,42
+3,97
+1,71
+2,41
+1,63
+2,15
+2,23


B
pdksA2-lacZ
120
80
40
0
Miller Units
0
1
2
3
4
5
Hours post induction

4.2.1. Supplementary Tables and Figures

Supplementary Table S1: Transcripts, which are up-regulated in strain PAO1Δ*nalA* (pJT*nalA*).

ORF[a]	Gene	Fold-change pJT*nalA* vs. pJT19[b]	Description[c]
PA0162		1,52	probable porin
PA0201		1,94	hypothetical protein
PA0277		1,60	conserved hypothetical protein
PA0284	*oscA*	3,77	hypothetical protein
PA0316	*serA*	1,50	D-3-phosphoglycerate dehydrogenase
PA0541		1,67	hypothetical protein
PA0578		1,57	conserved hypothetical protein
PA0579	*rpsU*	1,83	30S ribosomal protein S21
PA0582	*folB*	1,67	dihydroneopterin aldolase
PA0729		1,76	hypothetical protein
PA0760		1,51	conserved hypothetical protein
PA0922		1,76	hypothetical protein
PA1168		2,40	hypothetical protein
PA1169	*loxA*	1,93	probable lipoxygenase
PA1216		1,69	hypothetical protein
PA2204		1,56	probable binding protein component of ABC transporter
PA2911		1,51	probable TonB-dependent receptor
PA3360		1,58	probable secretion protein
PA3397	*fpr*	1,53	ferredoxin--NADP+ reductase
PA3446		1,98	conserved hypothetical protein

PA3450	*lsfA*	3,43	probable antioxidant protein
PA3600		1,62	conserved hypothetical protein
PA3601		1,85	conserved hypothetical protein
PA3742	*rplS*	1,56	50S ribosomal protein L19
PA3743	*trmD*	1,68	tRNA (guanine-N1)-methyltransferase
PA3744	*rimM*	1,92	16S rRNA processing protein
PA3745	*rpsP*	1,60	30S ribosomal protein S16
PA3817		1,54	probable methyltransferase
PA3894		1,59	hypothetical protein
PA3931		1,95	conserved hypothetical protein
PA3979		1,54	hypothetical protein
PA4063		1,78	hypothetical protein
PA4064		1,55	probable ATP-binding component of ABC transporter
PA4066		1,57	hypothetical protein
PA4139		1,87	hypothetical protein
PA4245	*rpmD*	1,52	50S ribosomal protein L30
PA4275	*nusG*	1,61	transcription antitermination protein NusG
PA4276	*secE*	1,55	secretion protein SecE
PA4326		1,54	hypothetical protein
PA4432	*rpsI*	1,69	30S ribosomal protein S9
PA4433	*rplM*	1,50	50S ribosomal protein L13
PA4481	*mreB*	1,50	rod shape-determining protein MreB
PA4563	*rpsT*	1,65	30S ribosomal protein S20

PA4567	*rpmA*	1,60	50S ribosomal protein L27
PA4635		1,59	conserved hypothetical protein
PA4748	*tpiA*	1,51	triosephosphate isomerase
PA4825	*mgtA*	1,63	Mg(2+) transport ATPase, P-type 2
PA4826		2,01	hypothetical protein
PA4836		1,60	hypothetical protein
PA4837		2,05	probable outer membrane protein
PA4940		1,50	conserved hypothetical protein
PA5049	*rpmE*	1,61	50S ribosomal protein L31
PA5072		1,55	probable chemotaxis transducer
PA5315	*rpmG*	1,86	50S ribosomal protein L33
PA5316	*rpmB*	1,84	50S ribosomal protein L28
PA5469		1,60	conserved hypothetical protein
PA5470		1,82	probable peptide chain release factor
PA5471		1,66	hypothetical protein
PA5532		1,71	hypothetical protein
PA5534		2,27	hypothetical protein
PA5535		2,42	conserved hypothetical protein
PA5536	*dksA2*	3,97	conserved hypothetical protein
PA5537		1,71	hypothetical protein
PA5538	*amiA*	2,41	N-acetylmuramoyl-L-alanine amidase

PA5540		2,15	hypothetical protein
PA5541	*pyrQ*	2,23	probable dihydroorotase
PA5570	*rpmH*	1,56	50S ribosomal protein L34
PA5539		1,63	hypothetical protein

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

b. The change means increase of the respective transcript levels in strain PAO1Δ*nalA* (pJT*nalA*) when compared with PAO1Δ*nalA* (pJT19).

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

Supplementary Table S2: Transcripts, which are down-regulated in the strain PAO1Δ*nalA* (pJT*nalA*).

ORF[a]	Gene	Fold-change pJT*nalA* vs. pJT19[b]	Description[c]
PA0758		- 1,51	hypothetical protein
PA1418		- 1,55	probable sodium:solute symport protein
PA1419		- 1,69	probable transporter
PA2375		- 1,51	hypothetical protein
PA2518	*xylX*	- 1,60	toluate 1,2-dioxygenase alpha subunit
PA4023		- 1,67	probable transport protein
PA4290		- 1,53	probable chemotaxis transducer
PA4306	*flp*	- 2,19	hypothetical protein
PA4551	*pilV*	- 1,54	type 4 fimbrial biogenesis protein PilV
PA4552	*pilW*	- 1,72	type 4 fimbrial biogenesis protein PilW
PA4553	*pilX*	- 1,50	type 4 fimbrial biogenesis protein PilX
PA4554	*pilY1*	- 1,57	type 4 fimbrial biogenesis protein PilY1
PA4659		- 1,86	probable transcriptional regulator
PA4660	*phr*	- 1,75	deoxyribodipyrimidine photolyase
PA4781		- 1,74	probable two-component response regulator
PA5082		- 1,58	probable binding protein component of ABC transporter
PA5083		- 1,50	conserved hypothetical protein
PA5116		- 1,59	probable transcriptional regulator

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

b. The change means increase of the respective transcript levels in strain PAO1Δ*nalA* (pJT*nalA*) when compared with PAO1Δ*nalA* (pJT19).

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

5. Appendix: The *Pseudomonas aeruginosa* Hfq-binding sRNA PhrD

INTRODUCTION

Filamentous phage are long, single-stranded DNA (ssDNA) viruses that infect Gram-negative Bacteria (Russel & Model, 2006). Unlike other phage, which lyse the host cell during release of new phage particles, filamentous phage progeny are constantly released while maintaining the vitality of their host, albeit at a reduced growth rate (Delbrück, 1946; Hoffmann-Berling & Mazé, 1964; Salivar *et al.*, 1964). After filamentous phage infection, the ssDNA forms a hairpin structure recognised by the host-encoded XerC recombinase (Val *et al.*, 2005), which mediates the insertion into the host genome (Huber & Waldor, 2002). Expression of prophage genes is tightly regulated (Waldor & Friedman, 2005) and occurs from the replicative form (RF) of the phage genome, a double-stranded DNA derived from the initial ssDNA or from prophage activation (Greenstain *et al.*, 1988; Horiuchi, 1997; Asano *et al.*, 1999). In *Vibrio cholerae*, activation of prophage CTXφ is triggered by the SOS response, after degradation of the host repressor protein LexA (Quinones *et al.*, 2005). LexA degradation abolished the sequestration of the phage-encoded transcriptional repressor RstR by the replicator protein RstA and the positive regulator RtsC (Davis *et al.*, 2002). Consequently, RtsA initiates replication of the positive strand using the integrate phage genome as template (McLeod *et al.*, 2005). As a specific type of symbiosis, some phage genes code for virulence factors or for resistance determinants (Waldor & Mekalanos, 1996; Webb *et al.*, 2004; Bille *et al.*, 2008). Filamentous phages can kill the host cell under specific circumstances (Kuo *et al.*, 1994; Davis *et al.*, 2002), and the derived progeny are capable of infecting and killing previously resistant neighbouring cells (Rice *et al.*, 2009). These superinfective phages are characterized by various mutations in one or more phage genes, typically leading to disruption of the hosts' membrane integrity (Bradley & Dewar, 1967; Schwartz & Zinder, 1968).

The Pf4 prophage of *Pseudomonas aeruginosa* is activated in a *mvaT/mvaU* double mutant (Li *et al.*, 2009), and superinfective phage particles are released either upon oxidative stress (Webb *et al.*, 2003) or from biofilms (Webb *et al.*, 2004; Rice *et al.*, 2009). The selective death of host cells as a consequence of the release of the superinfective Pf4 particles leads to diversification within the biofilm multicellular community (Rice *et al.*, 2009).

In a survey for novel sRNAs in PAO1, Sonnleitner and collaborators (2008) have isolated the sRNA PhrD through co-immunoprecipitation with Hfq. The *phrD* gene is located at the beginning of the Pf4 prophage genome (Stover *et al.*, 2000). In this study, attempts have been made to test whether PhrD can act as a *trans*-antisense regulatory RNA.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani medium (Miller, 1972) supplemented with appropriate antibiotics. Antibiotics were added to final concentrations of 50 µg gentamycin ml^{-1}, 400 µg kanamycin ml^{-1} and 125 µg tetracycline ml^{-1}, respectively.

RNA preparation and Northern-blot analyses. Total RNA from PAO1 harvested at the indicated OD_{600} or at a given time following toluic acid induction was purified using the hot phenol method (Lin-Chao and Bremer, 1986). For *phrD* expression in acidic medium, PAO1 was grown in LB at 37°C until the culture reached an OD_{600} of 1.0. The medium was then adjusted to pH 2 with HCl and the culture was further incubated at 37°C. The steady state levels of PhrD were determined by Northern-blot analyses using 10 µg total RNA. The RNA was denatured for 5 minutes at 65°C in loading buffer containing 50% formamide, separated on 8% polyacrylamide / 8M urea gels, and then transferred to nylon membranes by semi-dry electroblotting. The RNA was cross-linked to the membrane by exposure to UV light. The membranes were hybridized at 55°C with gene-specific ^{32}P-end labelled oligonucleotides (PhrD: P31, 5S rRNA: I26, Table 2), and the hybridisation signals were visualized using a PhosphoImager (Molecular Dynamics).

Construction of plasmid pME-p*tac-tolR::lacZ* and β-galactosidase assays. For construction of the translational p*tac-tolR*::*lacZ* fusion gene, the region comprising bp -174 to +24 with regard to the ATG codon of the *tolR* gene, was amplified by PCR using PAO1 genomic DNA together with forward primer B67 containing a *Bam*HI site and reverse primer C67 containing a *Pst*I site (Table 2). The PCR product was cloned into the corresponding sites of plasmid pME6014-p*tac*, resulting in pME-p*tac-tolR*::*lacZ*. Strains PAO1 and PAO1ΔPf4 har-

bouring pME-p*tac-tolR*::*lacZ* were grown in LB medium under aeration at 37°C. One millilitre of bacterial suspension was withdrawn at several ODs for determination of the β-galactosidase activity, which was determined as described (Miller, 1972). All experiments were done in triplicate.

Construction of plasmid pJT*phrD*. The plasmid pJT*phrD* bearing the *phrD* gene under the control of a toluic acid inducible promoter (*Pm* promoter) was constructed as follows. The *phrD* DNA sequence (bp +1 to + 149 with regard to the *phrD* transcriptional start site) was amplified by PCR using primers E56 (includes the *Pm* promoter sequence) and S51, containing an *Xba*I and *Kpn*I restriction site, respectively (Table 2). The PCR fragment was then cloned into the corresponding sites of plasmid pJT19 (Table 1), resulting in pJT*phrD*. PhrD synthesis was induced by addition of 2 mM toluic acid to cells growing in LB medium, at an OD_{600} of 0.2-0.6.

RESULTS AND DISCUSSION

PhrD is induced upon entry in stationary phase and upon acidic stress

The sRNAs PhrD, also named P20 was detected using a shotgun-cloning approach (RNomics) in combination with Hfq co-immunopreciptation (Sonnleitner *et al.*, 2008) as well as by bio-computation (Livny *et al.*, 2006; Gonzalez *et al.*, 2008). The *phrD* gene is the proximal-most gene of the Pf4 prophage, located between the circularization site and gene PA0715 (Figure 1A). The 72 nucleotides long RNA PhrD (Figure 1B) is weakly expressed throughout growth, and reaches a maximum in stationary phase (Figure 1C). Furthermore, transcription of *phrD* appears to depend on Hfq (Sonnleitner *et al.*, 2008). In addition, acid stress induces *phrD* expression (Figure 1D).

In *E. coli*, acid stress tolerance is generally enacted through the glutamate and arginine decarboxylase systems (Castanie-Cornet *et al.*, 1999; Richard & Foster, 2004; Bearson *et al.*, 2009). During stationary phase acid resistance, the stringent starvation protein A (SspA) inhibits the accumulation of the H-NS transcriptional inhibitor (Hansen *et al.*, 2005). The same protein SspA is involved in the transcriptional activation of bacteriophage P1 late genes, which enables the switch from a lysogenic to a lytic cycle (Williams *et al.*, 1991**;** Hansen *et al.*, 2003). In *P. aeruginosa*, the H-NS-like proteins MvaT and MvaU are both involved in arginine uptake and Pf4 silencing (Li *et al.*, 2009), and a microarray analysis of a PAO1 *mvaT* deletion mutant revealed that MvaT is necessary for phage gene PA0717 expression (Vallet *et al.*, 2004; Westfall *et al.*, 2006). As in the study of Vallet *et al.* (2004) the RNA purification method did not allow for the detection of transcripts smaller than 100 nucleotides, a possible impact of MvaT on *phrD* synthesis could not be delineated. However, a ChIP-on-chip analysis performed by Castang and collaborators (2008) revealed that both, MvaT and MvaU bind to the PAO1 chromosome loci PA0714 to PA0717, with a peak in the number of reads between PA0717 and PA0718. The latter analysis disclosed also a silencing function of MvaT

and MvaU on expression of the regulatory RNA RsmZ (Brencic *et al.*, 2009). Hence, the determination of the abundance of PhrD in PAO1 harbouring deletions in either *mvaT*, *mvaU* or both, could shed light on the mechanisms of *phrD* expression under acidic stress conditions (Figure 4). Finally, another ChIP-on-chip analysis performed by Wei and collaborators (2012) disclosed that the promoters of PA0717 and *phrD* are both bound by the H_2O_2-responsive transcriptional activator OxyR. This result is in agreement with previous studies, as the Pf4 phage is released upon accumulation of reactive oxygen species (ROS) in *P. aeruginosa* microcolonies (Webb *et al.*, 2003). Therefore, *phrD* induction might occur in response to different stresses (acid, ROS) by utilizing different regulators.

PhrD: open questions

The *phrD* gene is located near the *attL* site of the Pf4 prophage, 500 bp upstream of gene PA0715, encoding a putative reverse transcriptase (Figure 1A). The genome-wide random-insertion library (Jacobs *et al.*, 2003) does not contain insertion mutants in either *phrD* or PA0715, which could suggest that this region is pivotal for survival. This may explain why all attempts made in this study failed to delete *phrD* by double recombination of a suicide vector. In the Pf4 prophage a putative toxin-antitoxin (TA) module is present - the ParE-like toxin and the Phd-like antitoxin (prevents host death; Webb *et al.*, 2004). The Phd antitoxin is usually associated with the *doc* toxin (death on curing). It is conceivable that PhrD is somehow involved in repression of the Pf4 encoded TA module. As the deletion of *phrD* within the prophage was proven unsuccessful, a possibility to test this hypothesis would be to perform the deletion of *phrD* in the Pf4 replicative form, and then transform the PAO1ΔPf4 strain with the mutated Pf4 DNA.

Initial attempts to over-express *phrD* under its own promoter failed (Sonnleitner *et al.*, 2008). In this study, we attempted to over-express *phrD* upon toluic acid addition in strain

PAO1 (pJT*phrD*) (Figure 2A) and in strain PAO1ΔPf4 (pJT*phrD*) (Figure 2B). Following induction, no increase in the abundance of the expected 72 nt long PhrD was obtained in both cases. Rather, several longer transcripts were detected, spanning from ~ 170 nt to 300 nt in length, possibly deriving from a stalling in RNA polymerase transcription. Interestingly, only the ~ 170 nt fragment is present in both strains, PAO1 (pJT19) and PAO1 (pJT*phrD*) (Figure 2A/C). This observation could suggest that *phrD* does not have a Rho-independent terminator and that the 72 nucleotide PhrD RNA is the result of a processing event. Using the ARNold Web-server for prediction of Rho-independent terminators (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php), none was predicted downstream of *phrD* (+1 to + 279 with regard to the *phrD* transcriptional start site; data not shown).

A C-rich region downstream of *phrD*, +127 to + 253 with regard to the *phrD* transcriptional start site (Figure 1B and Figure 4A), could also be involved in *phrD* termination. The reverse primer S51 (Table 2) used for the construction of pJT*phrD* contained the downstream sequence up to the first two CCCC repeats (Figure 1B). As mentioned above, a PhrD transcript which is ~ 95 nt longer than the expected 72 nucleotides (Sonnleitner *et al.*, 2008) was detected. This longer PhrD transcript detected by Northern-blot analysis would terminate almost halfway of the C-rich sequence, which ends at + 190 nt with respect to *phrD* transcriptional start site. Moreover, Sonnleitner and collaborators (2008) used vector pME*phrD* in an effort to over-produce PhrD in PAO1, which was unsuccessful. The reverse primer Y32 used for construction of pME*phrD* contained none of the C-rich repeats (Figure 1B). It is therefore tempting to hypothesize that the 18 repeats are required for *phrD* termination, possibly through a Rho-dependent mechanism. The transcription terminator Rho is a homohexameric protein with ATP-dependent helicase activity (Brennan *et al.*, 1987). Upon binding to a nascent RNA, the ATPase activity of Rho is activated (Delagoutte & von Hippel, 2003), which stimulates unwinding of RNA-DNA duplexes (von Hippel & Delagoutte, 2001; Richardson, 2002) and translocation along the RNA towards the RNA polymerase (RNAP) at specific

pause sites. The interaction of Rho with the RNAP triggers the release of the nascent RNA fragment (Guarente, 1979; Yager & von Hippel, 1987). The general requirement for Rho binding site on nascent RNAs is its richness in C-residues (Allfano *et al.*, 1991; Guérin *et al.*, 1998). Finally, some Rho-terminated mRNAs are subsequently processed by specific exonucleases to generate stabilizing 3'ends. Examples are found in *E. coli* during maturation of *trp* mRNA, which is processed by RNase II after Rho-dependent termination (Mott *et al.*, 1985), and during formation of the sRNA RygD (SibD, Peters *et al.*, 2009), which is required for regulation of the *ibsD* gene (Fozo *et al.*, 2008). Thus, the 72 nt PhrD sRNA could result from processing of the longer transcript after Rho-dependent termination.

PhrD has no effect on its predicted target *tolR*

A bioinformatic analysis for the prediction of sRNA targets (Busch *et al.*, 2008) was used to search for putative targets of PhrD. The *tolR* gene was among possible targets, and the predicted interaction would encompass the *tolR* ribosome binding sites (Figure 3A). The PAO1 *tolR* gene is part of the *tolQRAB* operon, in which *tolR* is co-transcribed with *tolQ*, but not with *tolAB* (Dennis *et al.*, 1996). Required for maintaining the integrity of the *E. coli* outer membrane (Cascales *et al.*, 2007; Gerding *et al.*, 2007), the TolA, TolR and TolQ proteins are required for transport of group A colicins and for import of filamentous phage DNA (Russel *et al.*, 1988; Click & Webster, 1997, 1998; Lazzaroni *et al.*, 1999). The three proteins form a transmembrane channel. Phage recognition and docking are mediated by the TolA protein (Riechmann & Holliger, 1997; Lubkowski *et al.*, 1999). As TolR is involved in both, phage entrance and release of new phage particles, the possible regulation of *tolR* by PhrD was studied. A part of the 5' UTR and the first 8 codons of *tolR* were fused in frame to the *lacZ* gene, and the β-galactosidase activity conferred by the fusion protein was monitored throughout growth of PAO1 and PAO1ΔPf4, respectively (Figure 3B). Under the experimental condition used, PhrD synthesis in strain PAO1 did not impact on *tolR* translation (Figure 3B).

PhrD, a retroelement and superinfective Pf4 phage

The presence of a reverse transcriptase (RT) in the Pf4 genome is unique among lysogenic filamentous phages. RTs in Prokaryotes are contained within genomic loci named retroelements. Initially characterized in retrons (Lampson *et al.*, 1989; Lim & Maas, 1989), RT genes are also found in diversity generating retroelements (DGR; Doulatov *et al.*, 2004), Abi elements (abortive phage infection; Fortier *et al.*, 2005; Durmaz & Klaenhammer, 2007) and group II introns, which represent the majority of the RT subtypes (Kojima & Kanehisa, 2008; Simon & Zimmerly, 2008).

Synthetized from retroelements, multicopy small DNAs (msDNA) were discovered in myxobacteria (Yee *et al.*, 1984) as well as in some clinical strains of *E. coli* (Lampson *et al.*, 1989), and consist of a single-stranded DNA branched from an internal G residue of the RNA from which it is derived (Inouye & Inouye, 1992). The synthesis of msDNA is atypical: a long precursor RNA is initially transcribed, folding in a stable secondary structure that is used both as a primer for msDNA synthesis, and as a template to form the branch linkage between the RNA and DNA components, which base-pair with the 3´end of one another. The RNA template is then degraded by an RNase H domain usually present in the RT. A specific RT is essential for msDNA biosynthesis, and the mRNA for the reverse transcriptase itself is located immediately downstream of the precursor RNA for msDNA synthesis (Inouye & Inouye, 1992). While a function for these msDNAs has not been defined, their synthesis in *E. coli* leads to an increase in the mutation rate (Maas *et al.*, 1994) and seems to alter protein synthesis (Jeong & Lim, 2004). In *Salmonella*, msDNA subtly influences phage sensitivity (Rychlik *et al.*, 2001) and antibiotic resistance (Boyd *et al.*, 2000).

The PAO1 Pf4 prophage encodes a putative RT (PA0715) with 44% similarity to the RNA-directed DNA polymerase of the *E. coli* 2.4-kb retron Ec73 within prophage ΦR73 (Winsor *et al.*, 2011). As a P4-like prophage, ΦR73 requires a helper phage (e.g. P2) to pro-

duce new phage particles which display the characteristic lytic growth cycle (Inouye *et al.*, 1991). Curiously, a small ORF encoded in the intergenic region between PA0716 and PA0717 shows 42% homology to the sequence encoding the repressor C protein of phage P2 (Webb *et al.*, 2004). Moreover, from a recent report by McElroy *et al.* (2011), the lysogenic-to-lytic conversion of the Pf4 phage is linked to an increase in single nucleotide polymorphism (SNP) occurring within and upstream of the putative repressor C gene. Consequently, the presence of the RT gene 500 bp downstream of the *phrD* gene, in addition to the presence of a putative ORF encoding for an ortholog of repressor C protein raises the question as to whether PhrD is part of a retronphage in PAO1, the activation of which could be linked to an increase of the mutation rate in the superinfective Pf4 phage, which possess a lytic phenotype (Figure 4A). To date, the presence of msDNA in *P. aeruginosa* has not been reported. Our attempts to purify msDNA from an overnight culture of PAO1 in LB (following the method described in Birnboim & Doly, 1979) yielded no signal in an agarose gel (data not shown). Given that the Pf4 phage becomes superinfective when released from 5-to-7 days old biofilms (Webb *et al.*, 2004; Rice *et al.*, 2009), attempts to detect msDNA could be performed under these conditions.

As stated above, a C-rich short sequence repeat (SSR) was detected between *phrD* and PA0715, in which the heptameric sequence CCCCGCT is repeated 18 times (Stover *et al.*, 2000; Figure 1A and Figure 4A). The presence of the repeat sequence $(AGCGGGG)_{18}$ in the Pf4 template DNA strand raises further hypotheses with regard to its involvement in expression of either *phrD* or PA0715, or both. These G-rich repeats are potentially capable of forming a complex secondary structure known as G-quadruplex (Figure 4). The software QGRS Mapper for prediction of G-quadruplexes (Kikin *et al.*, 2006) predicted a total of 5 QGRS (quadruplex forming G-rich sequence) in the sequence between *phrD* and PA0715. Based on the stress-induced duplex destabilisation (SIDD) model of Benham (1992), the enhanced gyrase activity in exponential phase leads to a negatively supercoiled DNA; a similar phenome-

non that occurs during exposition to specific stresses, such as temperature, osmolarity, aerobic-to-anaerobic switch and oxidative stress (Rohde *et al.*, 1994; Weinstein-Fischer *et al.*, 2000; Hatfield & Benham, 2002; Cheung *et al.*, 2003; Salmon *et al.*, 2003). As a consequence, local non-B DNA motifs are formed to counteract and disperse the accumulated torsional energy. Under these conditions, single-stranded G-rich DNA sequences form stable G-tetraloops (Duquette *et al.*, 2004; Wang *et al.*, 2004). As non-B DNA motifs, G-quadruplexes control recombination, replication and gene regulation (reviewed in Hatfield & Benham, 2002). The formation of a putative G-quadruplex between *phrD* and PA0715 in either the ssDNA or in the RF form upon supercoiling stress could impact on the expression of these two genes. As the G-stretches are on the template strand of both genes, the G-quadruplex might represent an alternative terminator for *phrD*, as observed in other studies (Simonsson *et al.*, 1998; Siddiqui-Jain *et al.*, 2002). Moreover, G-quadruplexes located within promoters can alter the expression of the downstream genes (Benham, 1996; Sheridan *et al.*, 1998, 1999). Hence, the putative G-quadruplex forming on the coding strand could affect transcription of the reverse transcriptase gene PA0715 (Figure 4A).

Alternatively, the G-rich sequence might influence *phrD* termination if expressed from the Pf4 RF. Following infection, the infective ssDNA of *E. coli* phages f1 and M13 assumes locally a double-stranded conformation presenting a -35 and a -10 promoter motif (Higashitani *et al.*, 1996). This motif is recognized by the host RNA polymerase (RNAP) and a RNA primer fragment is transcribed from this "promoter" (Higashitani *et al.*, 1997). The presence of a G-rich sequence halts the RNAP and causes its dissociation from the template after back-tracking (Zenkin *et al.*, 2006). The resulting RNA is used as a primer for the synthesis of the complementary negative strand by the host DNA polymerase III. In the case of phage Pf4, the G-rich repeat sequence in the coding region could alternatively represents the signal for transcription termination of the RNA primer, which consequently could be PhrD itself (Figure 4B).

REFERENCES

Allfano, P., Rivellini, F., Limauro, D., Bruni, C. B. & Carlomagno, M. S. (1991). A consensus motif common to all Rho-dependent prokaryotic transcription terminators. *Cell* **64(3)**, 553-563

Asano, S., Higashitani, A. & Horiuchi, K. (1999). Filamentous phage replication initiator protein gpII forms a covalent complex with the 5' end of the nick it introduced. *Nucleic Acids Res* **27**, 1882-1889.

Bearson, B. L., Lee, I. S. & Casey, T. A. (2009). *Escherichia coli* O157 : H7 glutamate- and arginine-dependent acid-resistance systems protect against oxidative stress during extreme acid challenge. *Microbiology* **155(Pt 3)**, 805-812.

Benham, C. J. (1992). The energetics of the strand separation transition in superhelical DNA. *J Mol Biol* **225**, 835-847.

Benham, C. J. (1996). Duplex destabilization in superhelical DNA is predicted to occur at specific transcriptional regulatory regions. *J Mol Biol* **255**, 425-434.

Bille, E., Ure, R., Gray, S. J., Kaczmarski, E. B., McCarthy, N. D., Nassif, X., Maiden, M. C. & Tinsley, C. R. (2008). Association of a bacteriophage with meningococcal disease in young adults. *PLoS ONE* **3**, e3885.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7(6)**, 1513-1523.

.

Boyd, D. A., Peters, G. A., Ng, L. & Mulvey, M. R. (2000). Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica Typhymurium* DT104. *FEMS Microbiol Lett* **189(2)**, 285-291.

Bradley, D. E. & Dewar, C. A. (1967). Intracellular changes in cells of *Escherichia coli* infected with a filamentous bacteriophage. *J Gen Virol* **1**, 179-188.

Brencic, A., McFarland, K. A., McManus, H. R., Castang, S., Mogno, I., Dove, S. I. & Lory, S. (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 RNA. *Mol Microbiol* **73(3)**, 434-445.

Brennan, C. A., Dombroski, A. J. & Platt, T. (1987). Transcription termination factor Rho is an RNA-DNA helicase. *Cell* **48(6)**, 945-952.

Busch, A., Richter, A. S. & Backofen, R. (2008). IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. *Bioinformatics* **24**, 2849-2856.

Cascales, E., Buchanan, S. K., Duche, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S. & Cavard, D. (2007). Colicin biology. *Microbiol Mol Biol Rev* **71**, 158-229.

Castang, S., McManus, H. R., Keith, H., Turner, K. H. & Dove, S. L. (2008). H-NS family members function coordinately in an opportunistic pathogen. *Proc Natl Acad Sci USA* **105(48)**, 18947-18952.

Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F. & Foster, J. W. (1999). Control of acid resistance in *Escherichia coli*. *J Bacteriol* **181**, 3525-3535.

Cheung, K. J., Badarinarayana, V., Selinger, D. W., Janse, D. & Church, G. M. (2003). A microarray-based antibiotic screen identifies a regulatory role for supercoiling in the osmotic stress response of *Eschicheria coli*. *Genome Res* **13**, 206-215.

Click, E. M. & Webster, R. E. (1997). Filamentous phage infection: required interactions with the TolA protein. *J Bacteriol* **179**, 6464-6471.

Click, E. M. & Webster, R. E. (1998). The TolQRA proteins are required for membrane insertion of the major capsid protein of the filamentous phage f1 during infection. *J Bacteriol* **180**, 1723-1728.

Davis, B. M., Kimsey, H. H., Kane, A. V. & Waldor, M. K. (2002). A satellite phage-encoded antirepressor induces repressor aggregation and cholera toxin gene transfer. *EMBO J* **21**, 4240-4249.

Delagoutte, E. & von Hippel, P. H. (2003). Helicase mechanisms and the coupling of helicases within macromolecular machines. Part II: integration of helicases into cellular processes. *Q Rev Biophys* **36**, 1-69.

Delbrück, M. (1946). Bacterial viruses or bacteriophages. *Biol Rev* **21**, 30-40.

Dennis, J. J., Lafontaine, E. R. & Sokol, P. A. (1996). Identification and characterization of the *tolQRA* genes of *Pseudomonas aeruginosa. J Bacteriol* **178(24)**, 7059-7068.

Doulatov, S., Hodes, A., Dai, L., Mandhana, N., Liu, M., Deora, R., Simons, R. W., Zimmerly, S. & Miller, J. F. (2004). Tropism switching in *Bordetella* bacteriophage defines a family of diversity generating retroelements. *Nature* **431**, 476-481.

Duquette, M. L., Handa, P., Vincent, J. A., Taylor, A. F. & Maizels, N. (2004). Intracellular transcription of G-rich DNAs induces formation of G-loops, novel structures containing G4 DNA. *Genes Dev* **18(13)**, 1618-1629.

Durmaz, E. & Klaenhammer, T. R. (2007). Abortive phage resistance mechanism AbiZ speeds the lysis clock to cause premature lysis of phage-infected *Lactococcus lactis*. *J Bacteriol* **189(4)**, 1417-1425.

Fortier, L. C., Bouchard, J. D. & Moineau, S. (2005). Expression and site-directed mutagenesis of the lactococcal abortive phage infection protein AbiK. *J Bacteriol* **187**, 3721-3730.

Fozo, E. M., Kawano, M., Fontaine, F., Kaya, Y., Mendieta, K. S., Jones, K. L., Ocampo, A., Rudd, K. E. & Storz, G. (2008). Repression of small toxic protein synthesis by the Sib and OhsC small RNAs. *Mol Microbiol* **70(5)**, 1076-1093.

Gerding, M. A., Ogata, Y., Pecora, N. D., Niki, H. & de Boer, P. A. (2007). The *trans*-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in *E. coli*. *Mol Microbiol* **63**, 1008-1025.

Gonzalez, 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.

Greenstein, D., Zinder, N. D. & Horiuchi, K. (1988). Integration host factor interacts with the DNA replication enhancer of filamentous phage f1. *Proc Natl Acad USA* **85**, 6262-6266.

Guarente, L. (1979). Restoration of termination by RNA polymerase mutations is *rho* allele-specific. *J Mol Biol* **129**, 295-304.

Guérin, M., Robichon, N., Geiselmann, J. & Rahmouni, A. R. (1998). A simple polypyrimidine repeat acts as an artificial Rho-dependent terminator *in vivo* and *in vitro*. *Nucleic Acids Res* **26**, 4895-4900.

Hansen, A.-M., Lehnherr, H., Wang, X., Mobley, V. & Jin, D. J. (2003). *Escherichia coli* SspA is a transcription activator for bacteriophage P1 late genes. *Mol Microbiol* **48**, 1621-1631.

Hansen, A.-M., Qiu, Y., Yeh, N., Blattner, F. R., Durfee, T. & Jin, D. J. (2005). SspA is required for acid resistance in stationary phase by downregulation of H-NS in *Escherichia coli*. *Mol Microbiol* **56(3)**, 719-734.

Hatfield, G. W. & Benham, C. J. (2002). DNA topology-mediated control of global gene expression in *Escherichia coli*. *Annu Rev Genet* **36**, 175-203.

Heeb, S., Blumer, C. & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* **184**, 1046-1056.

Higashitani, N., Higashitani, A., Guan, Z. W. & Horiuchi, K. (1996). Recognition mechanisms of the minus-strand origin of phage f1 by *Escherichia coli* RNA polymerase. *Genes Cells* **1(9)**, 829-841.

Higashitani, A., Higashitani, N. & Horiuchi, K. (1997). Minus-strand origin of filamentous phage *versus* transcriptional promoters in recognition of RNA polymerase. *Proc Natl Acad Sci USA* **94**, 2909-2914.

Hoffmann-Berling, H. & Mazé, R. (1964). Release of male specific bacteriophages from surviving host Bacteria. *Virology* **22**, 305-313.

Horiuchi, K. (1997). Initiation mechanisms in replication of filamentous phage DNA. *Genes Cells* **2**, 425-432.

Huber, K. E. & Waldor, M. K. (2002). Filamentous phage integration requires the host recombinases XerC and XerD. *Nature* **417**, 656-659.

Inouye, M. & Inouye, S. (1992). Retrons and multicopy single-stranded DNA. *J Bacteriol* **174(8)**, 2419-2424.

Inouye, S., Sunshine, M. G., Six, E. W. & Inouye, M. (1991). Retronphage *phi* R73: an *E. coli* phage that contains a retroelement and integrates into a tRNA gene. *Science* **252(5008)**, 969-971.

Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C. & other authors (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100(24)**, 14339-14344.

Jeong, M. A. & Lim, D. (2004). A proteomic approach to study msDNA function in *Escherichia coli*. *J Microbiol* **42(3)**, 200-204.

Kikin, O., D'Antonio, L. & Bagga, P. S. (2006). QGRS Mapper: a web-based server for predicting G-quadruplexes in nucleotide sequences. *Nucleic Acids Res* **34 (Web Server issue)**, W676-W682.

Kojima, K. K. & Kanehisa, M. (2008). Systematic survey for novel types of prokaryotic retroelements based on gene neighborhood and protein architecture. *Mol Biol Evol* **25**, 1395-1404.

Kuo, T. T., Chiang, C. C., Chen, S. Y., Lin, J. H. & Kuo, J. L. (1994). A long lytic cycle in filamentous phage Cf1tv infecting *Xanthomonas campestris pv. citri*. *Arch Virol* **135**, 253-264.

Lampson, B. C., Sun, J., Hsu, M. Y., Vallejo-Ramirez, J., Inouye, S. & Inouye, M. (1989). Reverse transcriptase in a clinical strain of *Escherichia coli*: production of branched RNA-linked msDNA. *Science* **243(4894 Pt 1)**, 1033-1038.

Lazzaroni, J. C., Germon, P., Ray, M. C. & Vianney, A. (1999). The Tol proteins of *Escherichia coli* and their involvement in the uptake of biomolecules and outer membrane stability. *FEMS Microbiol Lett* **177(2)**, 191-197.

Li, C., Wally, H., Miller, S. J. & Lu, C.-D. (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)**, 6211-6218.

Lim, D. & Maas, W. K. (1989). Reverse transcriptase-dependent synthesis of a covalently linked, branched DNA–RNA compound in *E. coli* B. *Cell* **56**, 891-904.

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.

Lubkowski, J., Hennecke, F., Plückthun, A. & Wlodawer, A. (1999). Filamentous phage infection: crystal structure of g3p in complex with its coreceptor, the C-terminal domain of TolA. *Structure* **7(6)**, 711-722.

Maas, W. K., Wang, C., Lima, T., Zubay, G. & Lim, D. (1994). Multicopy single-stranded DNAs with mismatched base pairs are mutagenic in *Escherichia coli*. *Mol Microbiol* **14(3)**, 437-441.

Mott, J. E., Galloway, J. L. & Platt, T. (1985). Maturation of *Escherichia coli* tryptophan operon mRNA: evidence for 3' exonucleolytic processing after rho-dependent termination. *EMBO J* **4(7)**, 1887-1891.

McElroy, K., Luciani, F., Hui, J., Rice, S. & Thomas, T. (2011). Bacteriophage evolution drives *Pseudomonas aeruginosa* PAO1 biofilm diversification. *BMC Bioinformatics* **12(Suppl 11)**, A2

McLeod, S. M., Kimsey, H. H., Davis, B. M. & Waldor, M. K. (2005). CTXφ and *Vibrio cholerae*: exploring a newly recognized type of phage-host cell relationship. *Mol Microbiol* **57**, 347-356.

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

Peters, J. M., Mooney, R. A., Kuan, P. F., Rowland, J. L., Keles, S. & Landick, R. (2009). Rho directs widespread termination of intragenic and stable RNA transcription. *Proc Natl Acad Sci USA* **106(36)**, 15406-15411.

Quinones, M., Kimsey, H. H. & Waldor, M. K. (2005). LexA cleavage is required for CTX prophage induction. *Mol Cell* **17**, 291-300.

Rice, S. A., Tan, C. H., Mikkelsen, P. J., Kung, V., Woo, J., Tay, M., Hauser, A., McDougald, D., Webb, J. S. & other authors (2009). The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *ISME J* **3(3)**, 271-282.

Richard, H. & Foster, J. W. (2004). *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. *J Bacteriol* **186(18)**, 6032-6041.

Richardson, J. P. (2002). Rho-dependent termination and ATPases in transcript termination. *Biochim Biophys Acta* **1577**, 251-260.

Riechmann, L. & Holliger, P. (1997). The C-terminal domain of TolA is the coreceptor for filamentous phage infection of *E. coli*. *Cell* **90(2)**, 351-360.

Rohde, J. R., Fox, J. M. & Minnich, S. A. (1994). Thermoregulation in *Yersinia enterocolitica* is coincident with changes in DNA supercoiling. *Mol Microbiol* **12**, 187-199.

Russel, M. & Model, P. (2006). Filamentous phage. In The bacteriophages (Second Edition), R. C. Calendar, ed. (New York, Oxford University Press, Inc.), pp. 146-160.

Russel, M., Whirlow, H., Sun, T. P. & Webster, R. E. (1988). Low-frequency infection of F- bacteria by transducing particles of filamentous bacteriophages. *J Bacteriol* **170**, 5312-5316.

Rychlik, I., Sebkova, A., Gregorova, D. & Karpiskova, R. (2001). Low-molecular-weight plasmid of *Salmonella enterica* serovar *Enteritidis* codes for retron reverse transcriptase and influences phage resistance. *J Bacteriol* **183(9)**, 2852-2858.

Salivar, W. O., Tzagoloff, H. & Pratt, D. (1964). Some physical-chemical and biological properties of the rodshaped coliphage M13. *Virology* **24**, 359-371.

Salmon, K., Hung, S. P., Mekjian, K., Baldi, P., Hatfield, G. W. & Gunsalus, R. P. (2003). Global gene expression profiling in *Escherichia coli* K12. The effects of oxygen availability and FNR. *J Biol Chem* **278**, 29837-29855.

Schwartz, F. M. & Zinder, N. D. (1968). Morphological changes in *Escherichla coli* infected with the DNA bacteriophage fl. *Virology* **34**, 352-355.

Sheridan, S., Benham, C. J. & Hatfield, G. W. (1998). Activation of gene expression by a novel DNA structural transmission mechanism that requires supercoil-induced DNA duplex destabilization in an upstream activating sequence. *J Biol Chem* **273**, 21298-21308.

Sheridan, S., Benham, C. J. & Hatfield, G. W. (1999). Inhibition of DNA supercoiling-dependent transcriptional activation by a distant B-DNA to Z-DNA transition. *J Biol Chem* **274**, 8169-8174.

Siddiqui-Jain, A., Grand, C. L., Bearss, D. J. & Hurley, L. H. (2002). Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc Natl Acad Sci USA* **99(18)**, 11593-11598.

Simon, D. M. & Zimmerly, S. (2008). A diversity of uncharacterized reverse transcriptases in Bacteria. *Nucleic Acids Res* **36(22)**, 7219-7229.

Simonsson T, Pecinka P, Kubista M. (1998). DNA tetraplex formation in the control region of *c-myc*. *Nucleic Acids Res* **26(5)**, 1167-1172.

Smith, C., Heyne, S., Richter, A. S., Will, S. & Backofen, R. (2010). Freiburg RNA Tools: a web server integrating IntaRNA, ExpaRNA and LocARNA. *Nucleic Acids Res* **38 (Suppl)**, W373-W377.

Sonnleitner, E., Sorger-Domenigg, T., Madej, M. J., Findeiss, S., Hackermüller, J., Hüttenhofer, A., Stadler, P. F., Bläsi, U. & Moll, I. (2008). Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. *Microbiology* **154**, 3175-3187.

Stover, K. C., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J. & other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1: an opportunistic pathogen. *Nature* **406**, 959-964.

Val, M. E., Bouvier, M., Campos, J., Sherratt, D., Cornet, F., Mazel, D. & Barre, F. X. (2005). The single-stranded genome of phage CTX is the form used for integration into the genome of *Vibrio cholerae*. *Mol Cell* **19**, 559-566.

Vallet, I., Diggle, S. P., Stacey, R. E., Cámara, M., Ventre, I., Lory, S., Lazdunski, A., Williams, P. & Filloux, A. (2004). Biofilm formation in *Pseudomonas aeruginosa*: fimbrial cup gene clusters are controlled by the transcriptional regulator MvaT. *J Bacteriol* **186(9)**, 2880-2890.

von Hippel, P. H. & Delagoutte, E. (2001). A general model for nucleic acid helicases and their "coupling" within macromolecular machines. *Cell* **104(2)**, 177-190.

Waldor, M. & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910-1914.

Waldor, M. K. & Friedman, D. I. (2005). Phage regulatory circuits and virulence gene expression. *Curr Op Microbiol* **8**, 459-465.

Wang, H., Noordewier, M. & Benham, C. J. (2004). Stress-induced DNA duplex destabilization (SIDD) in the *E. coli* genome: SIDD sites are closely associated with promoters. *Genome Res* **14**, 1575-1584.

Webb, J. S., Lau, M. & Kjelleberg, S. (2004). Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **186(23)**, 8066-8073.

Webb, J. S., Thompson, L. S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., Givskov, M. & Kjelleberg, S. (2003). Cell death in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **185(15)**, 4585-4592.

Wei, Q., Le Minh, P. N., Dötsch, A., Hildebrand, F., Panmanee, W., Elfarash, A., Schulz, S., Plaisance, S., Charlier, D, & other authors (2012). Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nucleic Acids Res* **40(10)**, 4320-4333.

Weinstein-Fischer, D., Elgrably-Weiss, M. & Altuvia, S. (2000). *Escherichia coli* response to hydrogen peroxide: A role for DNA supercoiling, Topoisomerase I and FIS. *Mol Microbiol* **35**, 1413-1420.

Westfall, L. W., Carty, N. L., Layland, N., Kuan, P., Colmer-Hamood, J. A. & Hamood, A. N. (2006). *mvaT* mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon *mexEF-oprN*. *FEMS Microbiol Lett* **255**, 247-254.

Williams, M. D., Fuchs, J. A. & Flickinger, M. C. (1991). Null mutation in the stringent starvation protein of *Escherichia coli* disrupts lytic development of bacteriophage P1. *Gene* **109**, 21–30.

Winsor, G. L., Lam, D. K., Fleming, L., Lo, R., Whiteside, M. D., Yu, N. Y., Hancock, R. E. & Brinkman, F. S. (2011). Pseudomonas Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Res* **39**(Database issue):D596-600.

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

Yager, T. D. & von Hippel, P. H. (1987). Transcript elongation and termination in *Escherichia coli*. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, pp. 1241-1275. Edited by F. C. Neidhardt and others Washington, DC: American Society for Microbiology.

Yee, T., Furuichi, T., Inouye, S. & Inouye, M. (1984). Multicopy single-stranded DNA isolated from a Gram-negative bacterium, *Myxococcus xanthus*. *Cell* **38**, 203-209.

Zenkin, N., Naryshkina, T., Kuznedelov, K. & Severinov, K. (2006). The mechanism of DNA replication primer synthesis by RNA polymerase. *Nature* **439**, 617-620.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31(13),** 3406-3415.

Table 1: Bacterial strains and plasmids

Strain or Plasmid	Genotype/Relevant features	Reference or Origin
***P. aeruginosa* strains**		
PAO1	Wild-type	Rice *et al.* (2009)
PAO1ΔPf4	PAO1, Δ(Pf4): Gm^R	Rice *et al.* (2009)
***E. coli* strains**		
Top 10	F-, *mcrA*, D(*mrr-hsd*RMS-*mcr*BC),p80*lacZ*DM15 D*lacX*74, *deoR*, *recA1*, *araD*139D(*ara-leu*)7697, *galK*, *rpsL*(Str^R), *endA1*, *nupG*	Stratagene
Plasmids		
pJT19	Broad-host range *Pm* promoter expression vector, Kan^R	Winther-Larsen *et al*. (2000)
pJTphrD	pJT19 carrying *phrD* (toluic acid inducible promoter *Pm*), Kan^R	This study
pME6014	Cloning vector for translational *lacZ* fusions, Tc^R	Heeb *et al.* (2002)
pME6014Ptac	pME6014 derivative containing the 0,9 Kbp *tac* promoter inserted in the *Eco*RI/*Bam*HI sites, Tc^R	This study
pME-Ptac-tolR::lacZ	pME6014Ptac carrying a translational *tolR*::*lacZ* fusion (-174 to +24 bp with respect to the *lacZ* ATG start codon), Tc^R	This study

Table 2: DNA oligonucleotides used in this study

Name	Sequence (5´ to 3´end)*	Restriction site	Binding region †
H49	TTTT**CTGCAG**TGGTTGCTTCCCTGTT-CATC	*Pst*I	784717 - 784736
I49	TTTT**CGATCG**GTGATCATAAAGCAAC-CAGG	*Pvu*I	785494 - 785475
J49	AAAA**CGATCG**TACAGTCGTC-TAGTCTCCTG	*Pvu*I	785537 - 785556
K49	AAAA**GAATTC**AAAACTTACTAC-CTGCATGC	*Eco*RI	786245 - 786226
S51	AAAA**GGTACC**AGCGGGGAGCGGG-GATTTAC	*Kpn*I	785638 - 785619
E56	AAAA**TCTAGA**AAGGCCTACCCCT-TAGGCTTTATGCATATGCCAAGAC-TAGGAGCAGC	*Xba*I	785497 - 785517
O64	AGCAGCGCGATGAAGCAAT		797111 - 797129
P64	TAGAGGCCATTTGTGACTGGA		785538 - 785518
B67	AAAA**GGATCC**CGACGAGTTCCAGGC-CATCC	*Bam*HI	1054492 - 1054511
C67	AAAA**CTGCAG**TGCGCTTGT-GACGAACCCTTGCC	*Pst*I	1054590- 1054568

* Restriction sites are in boldface and the promoter sequence of the *Pm* promote is underlined.

† The numbers indicate the complementary segments on PAO1 chromosome according to the *Pseudomonas* genome database http://www.pseudomonas.com

FIGURE LEGENDS

Figure 1. (A) Genetic organization of the Pf4 prophage in PAO1 (Winsor *et al.*, 2009). The *phrD* locus is depicted by a red arrow. Green arrows represent the Pf4 prophage genes, while genes outside the prophage are indicated in light purple. The black squares indicate the left (attL) and right (*attR*) attachment site. The black triangle between *phrD* and PA0715 symbolizes the short heptameric sequence TCCCCGC repeats. The annotated function of some genes is specified underneath the respective arrow. (B) Upstream and downstream sequences of *phrD* and putative secondary structure of PhrD. Left, the *phrD* gene is indicated in red. The putative -35 and -10 boxes of the σ^{70} dependent promoter are underlined. The downstream 18 repeats of the heptameric CCCCGCT sequence are shown in grey. The putative sites of transcription termination and processing are indicated by vertical plain and open arrows, respectively. The location of reverse primers Y32 and S51 used to construct plasmids pME*phrD* (Sonnleitner *et al.*, 2008) and pJT*phrD*, respectively, are indicated by horizontal arrows. Right, PhrD putative secondary structure as predicted by the MFold algorithm (Zuker, 2003). (C) Detection of PhrD RNA during growth by Northern-blot analysis (see Materials and Methods). A second RNA band with a size of ~ 170 nucleotides was also detected with the same probe. RNA markers (M) are shown at the left. (D) Influence of acidic pH on *phrD* expression. The PAO1 strain was grown in LB medium to an OD_{600} of 0,5. The pH was then adjusted to 2.0 and PhrD abundance was monitored by Northern blot analysis at different times thereafter.

Figure 2. Assessment of toluic acid-dependent induction of PhrD synthesis in PAO1 (A) or in PAO1ΔPf4 (B) carrying pJT*phrD*. Strains PAO1 and PAO1ΔPf4 carrying plasmid pJT19 were used as a control. The strains were grown in LB medium at 37°C until they reached an

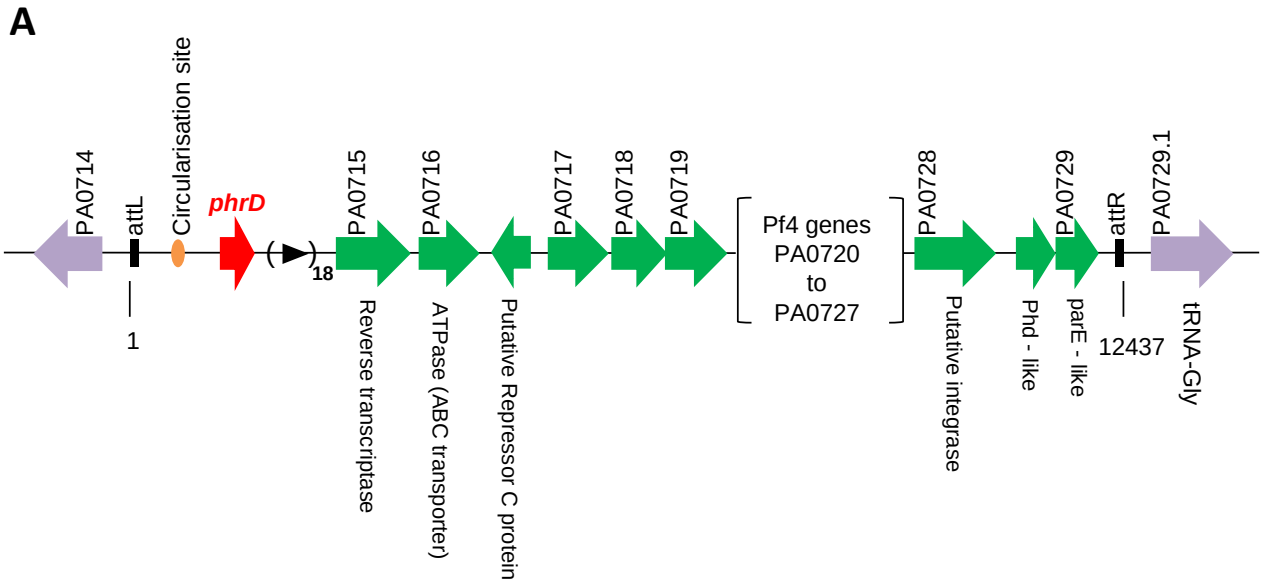
OD_{600} of 0.7 (time 0 minutes). Toluic acid was then added to the bacterial suspension. Samples were harvested at the indicated times post-induction and total RNA from these samples was used for Northern-blot analysis to monitor the steady-state levels of PhrD (see Materials and Methods).

Figure 3. PhrD does not influence translation of *tolR*. (A) Complementarity between the *tolR* mRNA and the PhrD RNA as predicted by the IntaRNA algorithm (http://rna.informatik.uni-freiburg.de:8080/IntaRNA.jsp; Busch *et al.*, 2008; Smith *et al.*, 2010). The putative Shine-Dalgarno sequence (SD) of the *tolR* mRNA is boxed in red. Possible interaction sites of PhrD with the *tolR* mRNA are indicated. (B) Left, schematic representation of the *tolR*::*lacZ* translational fusion. Transcription of the *tolR*::*lacZ* gene is driven by the *tac* promoter. Right, translation of the *tolR*::*lacZ* fusion was monitored by measuring the β-galactosidase activity in PAO1 (filled symbol) and PAO1ΔPf4 (open symbol) as a function of growth in LB medium. The experiment was performed in triplicate. Error bars, SD.

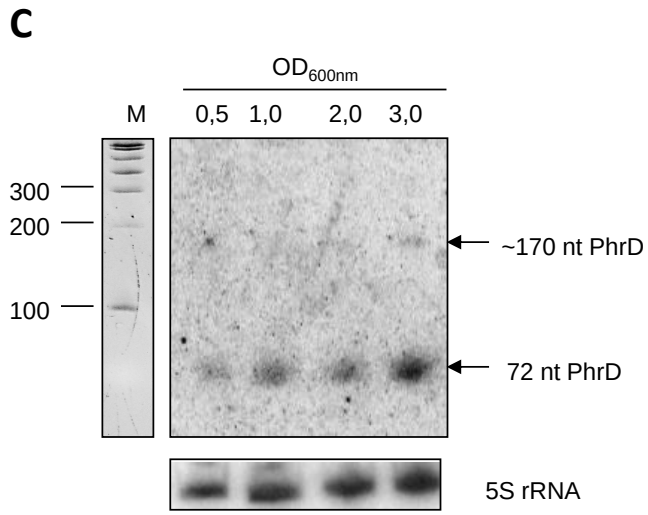
Figure 4. Hypotheses for PhrD function in PAO1. Dashed lines indicate hypothetical effects. (A) *phrD* transcription from PAO1 chromosome is induced during stationary phase of growth or during acidic or oxidative stresses. As *phrD* seemingly lacks a Rho-independent terminator, transcription termination might occur at the C-rich sequence downstream, and might require the terminator factor Rho. Eventually, the G-rich repeats on the complementary strand may form a G-quadruplex, which in turn could determine *phrD* termination and/or alter expression of the downstream PA0715 gene. Considering that *phrD* is located upstream of the PA0715 reverse transcriptase gene, the involvement of this enzyme in PhrD function can be as well envisioned, similarly as in other characterized retronphage systems (Yee *et al.*, 1984;

Lampson *et al.*, 1989; Inouye *et al.*, 1991). As retroelements can create genetic diversity, it may impact on the accumulation of mutations in the genome of the superinfective Pf4 phage (McElroy *et al.*, 2011). (B) Putative *phrD* transcription from the single stranded phage Pf4 replicating form (RF). Prophage Pf4 induction initiates with the synthesis of the (-) strand of the RF form (plain circle). In the presence of a compatible σ^{70}-dependent promoter on the single-stranded RF DNA, *phrD* is transcribed by the host RNA polymerase (RNAP) and terminates at the G-rich sequence. After the formation of a PhrD-RF duplex, PhrD might be used as an RNA template for the synthesis of the complementary (+) strand (dashed semi-circle) of Pf4 RF by the host DNA polymerase III (DNAP III). ↓: induction; ⊥: repression.

Figure 1


A
PA0714
attL
1
Circularisation site
phrD
18
PA0715
Reverse transcriptase
PA0716
ATPase (ABC transporter)
Putative Repressor C protein
PA0717
PA0718
PA0719
Pf4 genes
PA0720
to
PA0727
PA0728
Putative integrase
PA0729
Phd - like
parE - like
attR
12437
PA0729.1
tRNA-Gly


B
TTTTCGACAGGTCCTGCATGAGGATTGCATTTTTATCCTGGTTGCT
+ 1
-35
TTATGATCACCTTATGCCAAGACTAGGAGCAGCTCCAGTCACAAAT
-10
GGCCTCTACAGTCGTCTAGTCTCCTGTTTACGTTTGTGTTGCCCTT
Y32
TAGGCTCGGCTCGGGCTCGATGCCTAAAGGGCTTCACAAATAGTA
AATCCCCGCTCCCCGCTCCCCGCTCCCCGCTCCCCGCTCCCCGC
S51
TCCCCGCTCCCCGCTCCCCGCTCCCCGCTCCCCGCTCCCCGCTC
CCCGCTCCCCGCTCCCCGCTCCCCGCTCCCCGCTCCCCGCTCCC
TCGATCTTTTACTCATCCCCTCTGCTCTGGGTCTACCGTTTTT


C
OD600nm
M
0,5
1,0
2,0
3,0
300
200
100
~170 nt PhrD
72 nt PhrD
5S rRNA

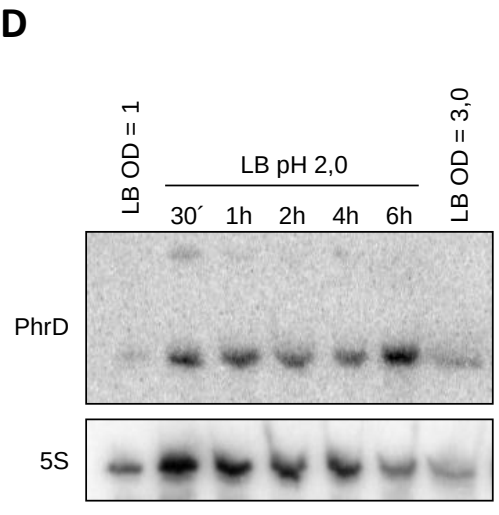
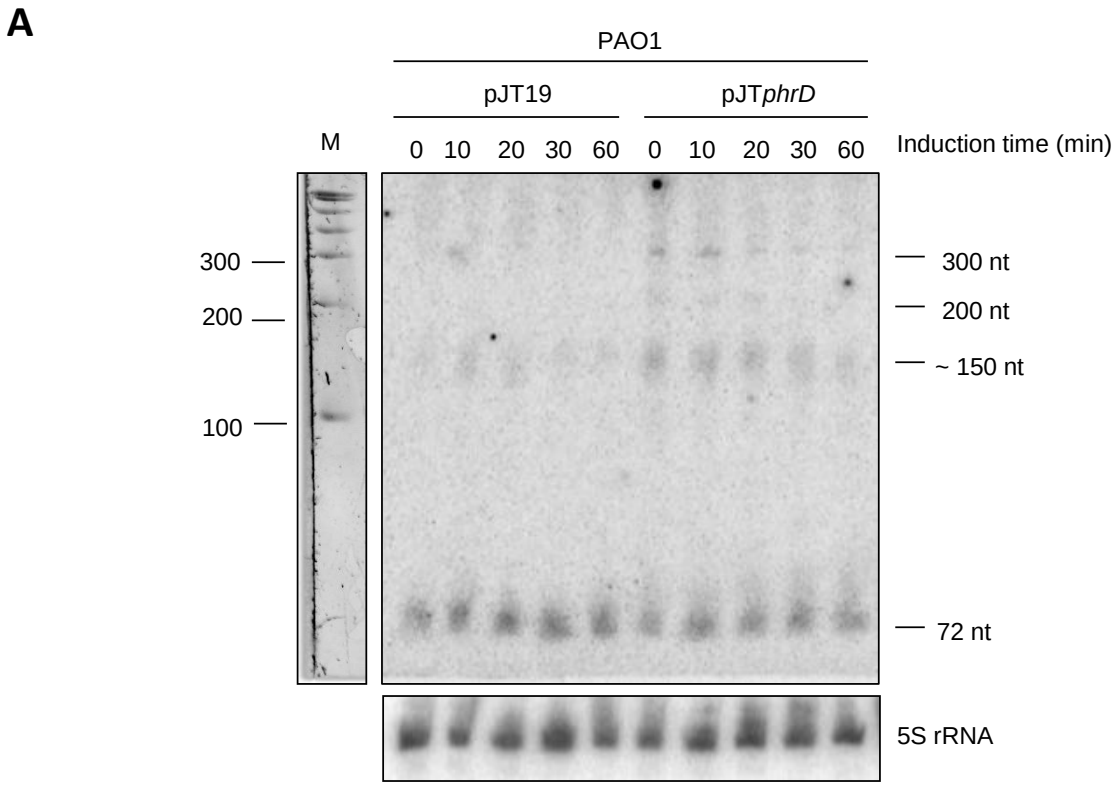

D
LB OD = 1
LB pH 2,0
LB OD = 3,0
30´
1h
2h
4h
6h
PhrD
5S

Figure 2


A
PAO1
pJT19
pJTphrD
M
0 10 20 30 60 0 10 20 30 60
Induction time (min)
300
200
100
300 nt
200 nt
~ 150 nt
72 nt
5S rRNA

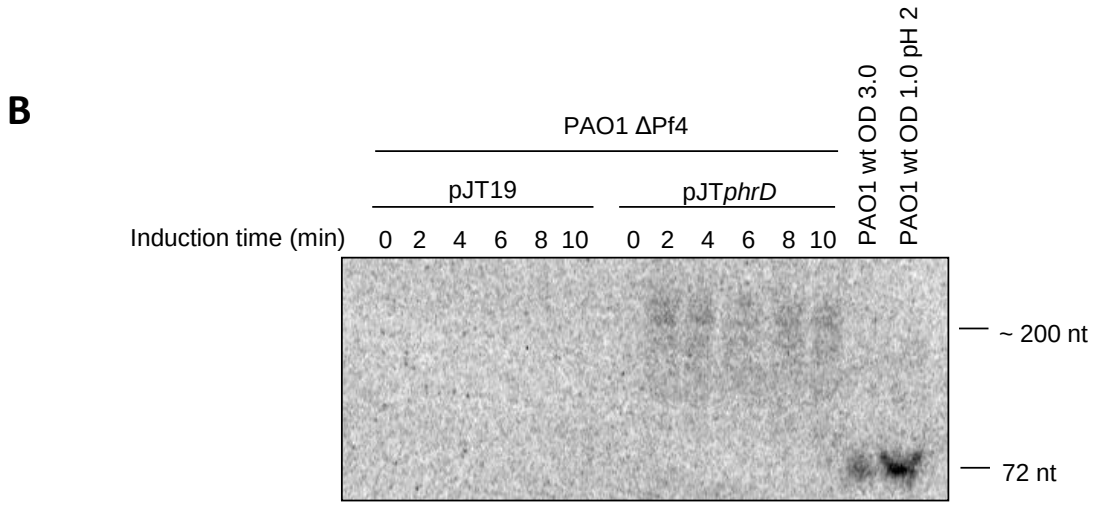
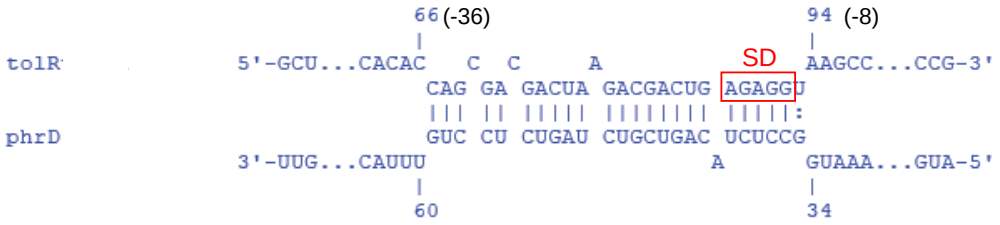
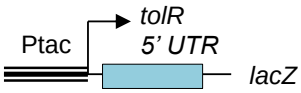

B
PAO1 ΔPf4
pJT19
pJTphrD
PAO1 wt OD 3.0
PAO1 wt OD 1.0 pH 2
Induction time (min)
0 2 4 6 8 10 0 2 4 6 8 10
~ 200 nt
72 nt

Figure 3

A


66 (-36)
94 (-8)
tolR
5'-GCU...CACAC C C A
SD
AAGCC...CCG-3'
CAG GA GACUA GACGACUG AGAGGU
GUC CU CUGAU CUGCUGAC UCUCCG
phrD
3'-UUG...CAUUU A
GUAAA...GUA-5'
60
34

B


Ptac
tolR
5' UTR
lacZ

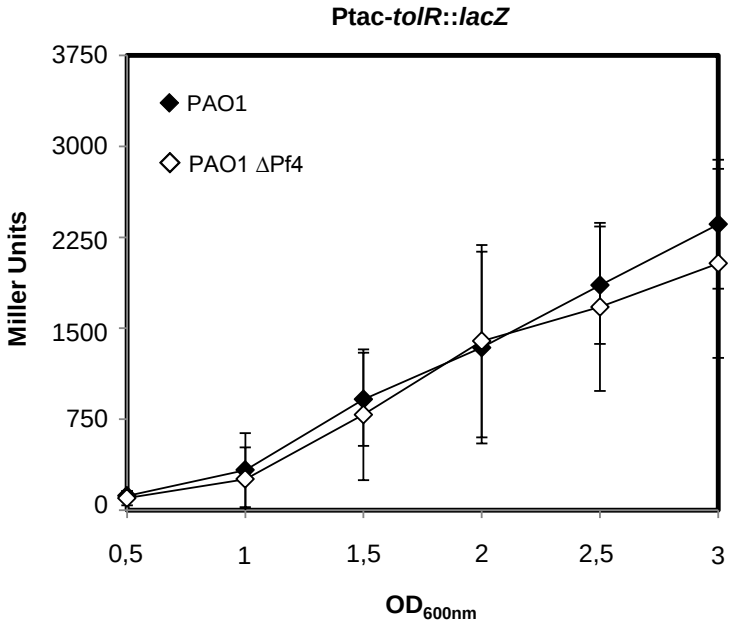
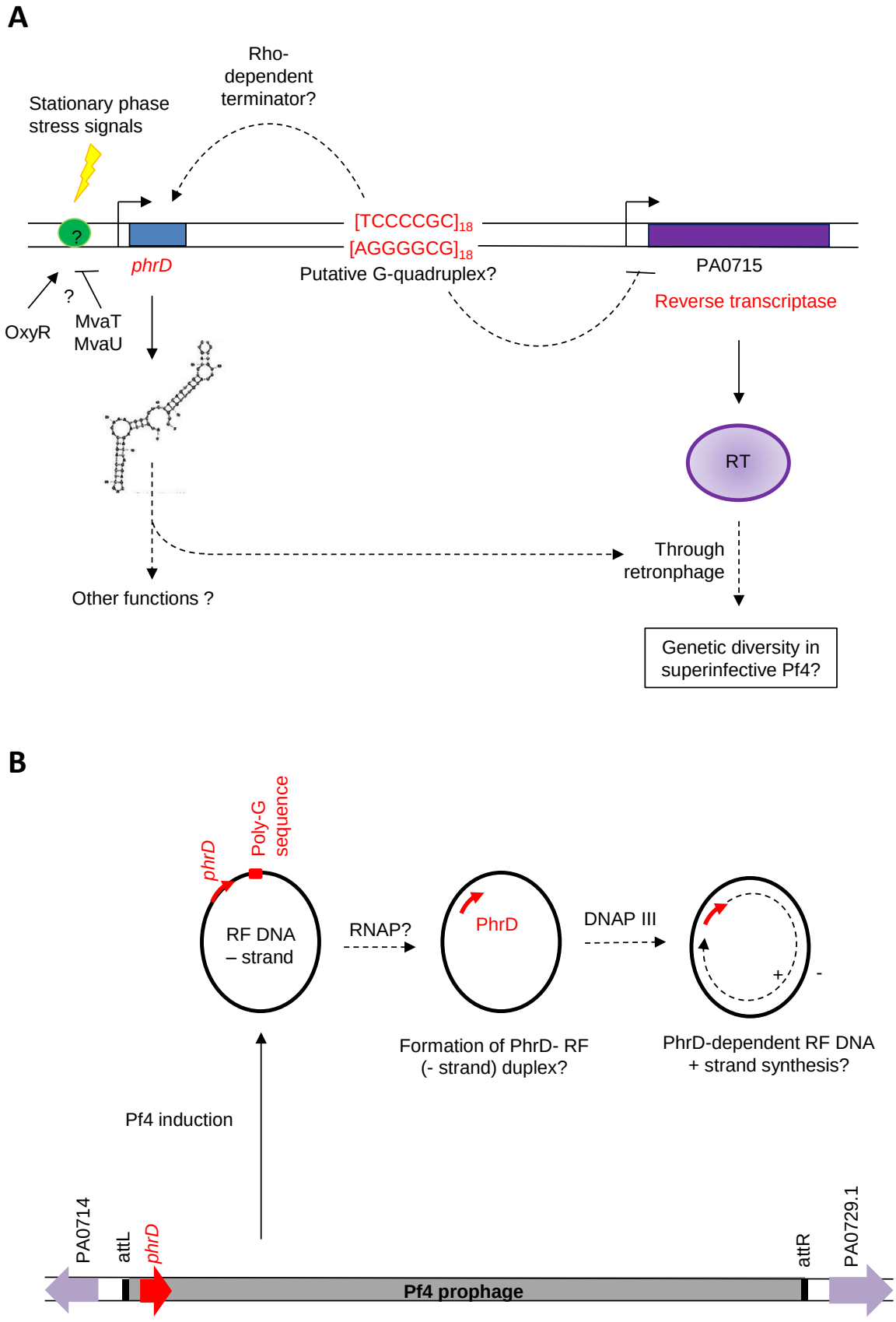

Ptac-tolR::lacZ
Miller Units
3750
3000
2250
1500
750
0
PAO1
PAO1 ΔPf4
0,5
1
1,5
2
2,5
3
OD600nm

Figure 4


A
Rho-dependent terminator?
Stationary phase stress signals
?
[TCCCCGC]18
[AGGGGCG]18
phrD
Putative G-quadruplex?
PA0715
Reverse transcriptase
?
OxyR
MvaT
MvaU
RT
Through retronphage
Other functions ?
Genetic diversity in superinfective Pf4?
B
phrD
Poly-G sequence
RF DNA – strand
RNAP?
PhrD
DNAP III
+
-
Formation of PhrD- RF (- strand) duplex?
PhrD-dependent RF DNA + strand synthesis?
Pf4 induction
PA0714
attL
phrD
attR
PA0729.1
Pf4 prophage

6. CURRICULUM VITAE

Personal Information

Name	Alessandra Romeo
Address	Kölblgasse 35/12
	1030 Vienna
	Austria
Date and Place of Birth	07.12.1983 in São Paulo (Brazil)
Nationality	Italian

Education

Doctoral Degree	University of Vienna 2008-2012
	Field of Study: Molecular Microbiology; Title of Study: "Characterisation of the regulatory RNA NalA in *Pseudomonas aeruginosa*. "
	Supervisor: Prof. U. Bläsi.
Master Degree	University of Pisa 2005-2007
	Field of study: RNA interference; Title of Study: "MicroRNA miR-100 and miR-125b over-expression inhibit the expression of transcription repressor LRF in Mouse Embryonic Fibroblasts".
	Supervisor: Prof. G. Rainaldi.
Bachelor Degree	University of Pisa 2002-2005
	Field of study: Developmental Biology; Title of Study: "Characterisation of thyroid hormone regulated genes in *Xenopus laevis* retina"
	Supervisors: Prof. S. Casarosa and Prof. M. Andreazzoli.
2002	Baccalauréat Scientifique
1997 - 2002	Lycée Français Jean Monnet, Bruxelles, Belgium
1992 - 1996	Lycée Chateaubriand, Rome, Italy
1988 - 1991	Ecole active bilingue Jeannine Manuel Paris, France

7. ACKNOWLEDGEMENTS

First and foremost, I am deeply grateful to my supervisor, Prof. Udo Bläsi, for his wise, patient and encouraging supervision during my 4 years PhD, and for his help during the writing of my article and my thesis.

I wish to thank all the people of the Bläsi and Moll Laboratories, for their caring support and enlightening scientific discussions. In particular, I owe all of my knowledge about microbiological techniques to Dr. Theresa Sorger-Domenigg, who patiently taught me everything from scratch, as I had no previous experience in the field. I am also grateful to Dr. Elisabeth Sonnleitner, without whom the construction of the PAO1Δ*nalA* mutant and the establishment of the role of NalA as an operon leader would not be possible. To Dr. Vesper and Dr. Märtens, I am thankful for their valuable suggestions and constructive feedback. In particular, my thanks go to Birgit Märtens for helping me in the English-to-German translation of the summary.

For her help in providing the method for mapping of *nalA* 5' and 3' ends, I owe gratitude to Dr. Karine Gonzalez, a former PhD student in the laboratory of Dr. Emmanuelle Charpentier.

For their help and collaboration with the microarray experiment and analysis, my thanks go to Dr. Karine Lapouge and Mélanie Dupasquier from the Université de Lausanne (Switzerland).

Finally, as part of the RNA-DK (RNA Doktoratskolleg) PhD program, I would like to thank the head of the program Prof. Andrea Barta, Prof. Renée Schroeder, Nicola Wiskocil and everyone who participated in the program, for their enthusiastic commitment and eclectic discussions.