Environmental Microbiology (2014) **16**(4), 1053–1068 doi:10.1111/1462-2920.12272

NrsZ: a novel, processed, nitrogen-dependent, small non-coding RNA that regulates *Pseudomonas aeruginosa* PAO1 virulence

Nicolas Wenner,[1] Alexandre Maes,[1] Marta Cotado-Sampayo[2] and Karine Lapouge[1]*
[1]*Department of Fundamental Microbiology, University of Lausanne, Lausanne CH-1015, Switzerland.*
[2]*Fasteris SA, Ch. du Pont-du-Centenaire 109, Case postale 28, Plan-les-Ouates CH-1228, Switzerland.*

Summary

The opportunistic pathogen *Pseudomonas aeruginosa* PAO1 has a remarkable capacity to adapt to various environments and to survive with limited nutrients. Here, we report the discovery and characterization of a novel small non-coding RNA: NrsZ (nitrogen-regulated sRNA). We show that under nitrogen limitation, NrsZ is induced by the NtrB/C two-component system, an important regulator of nitrogen assimilation and *P. aeruginosa's* swarming motility, in concert with the alternative sigma factor RpoN. Furthermore, we demonstrate that NrsZ modulates *P. aeruginosa* motility by controlling the production of rhamnolipid surfactants, virulence factors notably needed for swarming motility. This regulation takes place through the post-transcriptional control of *rhlA*, a gene essential for rhamnolipids synthesis. Interestingly, we also observed that NrsZ is processed in three similar short modules, and that the first short module encompassing the first 60 nucleotides is sufficient for NrsZ regulatory functions.

Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* PAO1 has a remarkable capacity to adapt to various environments and to survive with limited nutrients. To ensure an optimal coordination of genes involved in nutrient utilization, this versatile bacterium uses two-component systems, such as NtrB/C and CbrA/B, working in concert with the alternative sigma factor RpoN. RpoN is a global regulator involved in nitrogen metabolism, carbon assimilation, nutrient transport, motility, mucoidy and quorum sensing (Potvin *et al.*, 2008). The RpoN RNA holoenzyme binds to specific promoters of target genes with the consensus sequence T**GG**$_{-24}$CAC-N5-TT**GC**$_{-12}$W (bold: invariant nucleotides, W = A or T) upstream of the transcription start site (Barrios *et al.*, 1999). The alternative sigma factor RpoN is required by two-component systems, such as NtrB/C, to activate the transcription of target genes.

The two-component system NtrB/NtrC is an important regulator of nitrogen assimilation (Merrick and Edwards, 1995; Ninfa *et al.*, 1995; Li and Lu, 2007; Zhang and Rainey, 2008; Hervás *et al.*, 2009; Yeung *et al.*, 2009). Under nitrogen-limited conditions, the intracellular level of glutamine decreases and NtrB histidine kinase activity is enhanced, leading to its autophosphorylation. Activated NtrB transfers a phosphate group to its response regulator NtrC, which binds to specific upstream activating sequences, thereby activating transcription, with the alternative sigma factor RpoN, of its target genes (Kustu *et al.*, 1989). By contrast, the intracellular glutamine level increases under nitrogen excess, promoting NtrB phosphatase activity and leading to its auto-dephosphorylation (Dixon and Kahn, 2004). Until now, little is known about the NtrB/C network controlling *P. aeruginosa* metabolism (Nishijyo *et al.*, 2001; Li and Lu, 2007). In *Pseudomonas putida*, a transcriptome analysis in response to nitrogen availability was performed, which revealed that NtrB/C regulates various genes, such as *glnA* (encoding a glutamine synthetase), porin genes, amino acid transporter genes, and genes involved in urea assimilation and in carbon catabolism (Hervás *et al.*, 2008). Furthermore, it was shown that NtrB/C is also involved in the control of *P. aeruginosa* swarming motility, a surface movement (Merrick and Edwards, 1995; Ninfa *et al.*, 1995; Li and Lu, 2007; Zhang and Rainey, 2008; Hervás *et al.*, 2009; Yeung *et al.*, 2009).

Pseudomonas aeruginosa swarming motility is a social behaviour considered to be a virulence trait that is augmented under nitrogen limitation and in response to certain amino acids, in turn leading to an increase in *P. aeruginosa* resistance to multiple antibiotics (Köhler

Received 25 April, 2013; revised 26 August, 2013; accepted 28 August, 2013. *For correspondence. E-mail karine.lapouge@unil.ch; Tel. (+41) (0) 21 692 5601; Fax (+41) (0) 21 692 5605.

et al., 2000; Overhage *et al*., 2008). Furthermore, conditions for swarming are similar to those found in the lung, an organ that can be infected by *P. aeruginosa* (Hutchison and Govan, 1999; Breidenstein *et al*., 2011). To swarm, the bacterium requires flagella, type IV pili and rhamnolipids production (Köhler *et al*., 2000; Déziel *et al*., 2003; Caiazza *et al*., 2005; Overhage *et al*., 2007). Rhamnolipids are tension-active glycolipid molecules, and their biosynthesis is mediated by the *rhlAB* operon encoding RhlA and RhlB rhamnosyltransferases (Ochsner *et al*., 1994; Soberón-Chávez *et al*., 2005). The swarming motility of the *P. aeruginosa rhlA* negative strain is impaired, whereas an *rhlB* negative strain is able to swarm (Köhler *et al*., 2000; Déziel *et al*., 2003). Interestingly, rhamnolipids production is stimulated under unfavourable nutrient conditions, and availability of carbon, nitrogen and phosphate in particular was shown to modulate the production of these surfactants (Lang and Wullbrandt, 1999; Clarke *et al*., 2010).

Noteworthy, bacteria can also modulate their cellular functions at the post-transcriptional level using small non-coding RNAs (sRNAs). As regulatory molecules, sRNAs have two major advantages: they are synthesized at low energy cost for the cell and they act rapidly. sRNAs can be divided mainly into two classes: the first class includes sRNAs that capture RNA-binding proteins [e.g. RsmA/CsrA type (Lapouge *et al*., 2008)], while the second class of sRNAs undergo base-pairing interactions with target mRNAs, resulting in the regulation of translation and/or mRNA degradation/stabilization and often requires the RNA chaperone Hfq for function. The Hfq chaperone is an abundant, stable and hexameric protein, which was originally identified as a host factor necessary for the replication of bacteriophage Qβ in *Escherichia coli* (Franze de Fernandez *et al*., 1972). In *Enterobacteriaceae*, Hfq facilitates the interaction of regulatory sRNAs with their mRNAs targets and is important for the stability of the sRNAs (Storz *et al*., 2011). Interestingly, in *P. aeruginosa*, Hfq was shown to be a global regulator of virulence and stress response, and to co-immunoprecipitate, bind and stabilize sRNAs, but no evidence has been presented so far for its involvement in the sRNAs–mRNAs interaction (Sonnleitner *et al*., 2003; 2006; 2008). Extensive studies carried out in *E. coli* have shown the existence of around 100 sRNAs, representing around 2% of protein-coding genes in this bacterium (Sharma and Vogel, 2009; Waters and Storz, 2009). Until recently, only 44 sRNAs were reported in *P. aeruginosa*, of which only a few were experimentally validated and functionally characterized (Sonnleitner *et al*., 2010). These sRNAs were shown to play a crucial role in the regulation of primary and secondary metabolism (Sonnleitner and Haas, 2011; Sonnleitner *et al*., 2012). New studies using RNA sequencing technology have led to the identification of over 500 novel transcripts in *P. aeruginosa*, among which 50 were validated (Dötsch *et al*., 2012; Ferrara *et al*., 2012; Gómez-Lozano *et al*., 2012).

To discover novel sRNAs regulating the adaptation of *P. aeruginosa* to its nutritional environment, we conducted a bioinformatics-based approach and discovered a novel sRNA that we named NrsZ (nitrogen-regulated sRNA). We showed that NrsZ is highly conserved among Pseudomonads, and its induction in PAO1 under nitrogen-limited condition is regulated by the two-component system NtrB/C in concert with the alternative sigma factor RpoN. We demonstrated that NrsZ modulates *P. aeruginosa* motility by controlling the production of rhamnolipid surfactants, virulence factors notably needed for swarming motility. This regulation takes place through the post-transcriptional control of *rhlA*, a gene essential for rhamnolipids synthesis. Furthermore, we observed that NrsZ is processed to yield three similar short modules, and that the first 60 nucleotides (nt) encoding the first module are sufficient to ensure regulatory functions.

Results

Identification of NrsZ, a nitrogen-dependent, non-coding small RNA

To discover novel sRNAs involved in the regulation of nitrogen source utilization by *Pseudomonas aeruginosa* PAO1, we used a bioinformatics approach and searched for putative sRNAs regulated by the alternative sigma factor RpoN, which is required for the activation of target genes by the two-component system NtrB/C. Hence, we used the pattern search tool Fuzznuc (Rice *et al*., 2000) to query 162 intergenic regions of the PAO1 genome described by González *et al*. (González *et al*., 2008) to encode putative sRNAs using the RpoN consensus sequence (T**GG**CAC-N_5-TT**GC**W) based on Barrios *et al*. (Barrios *et al*., 1999). Twelve intergenic regions containing conserved RpoN consensus sequences were identified. As expected, the RpoN consensus sequence of the described sRNA CrcZ and the RNA NalA were also found (Sonnleitner *et al*., 2009; Romeo *et al*., 2012).

One intergenic region was chosen for further investigation due to the prediction of the well-conserved RpoN consensus sequence T**GG**$_{-24}$CACAGCCCCT**GC**$_{-12}$A, and its position between the tail-to-tail genes *ntrC* and PA5126 (Fig. S1). To investigate the transcriptional activity of the predicted RpoN promoter of a putative sRNA located in the *ntrC*-PA5126 intergenic region, we measured β-galactosidase activity of the chromosomal *sRNA-lacZ* transcriptional reporter fusion inserted into the PAO1 wild-type (WT) strain (PAO6850). The promoter cloned in this fusion encompassed the predicted –309 to +1 region relative to the start site of transcription. Then, regulation of

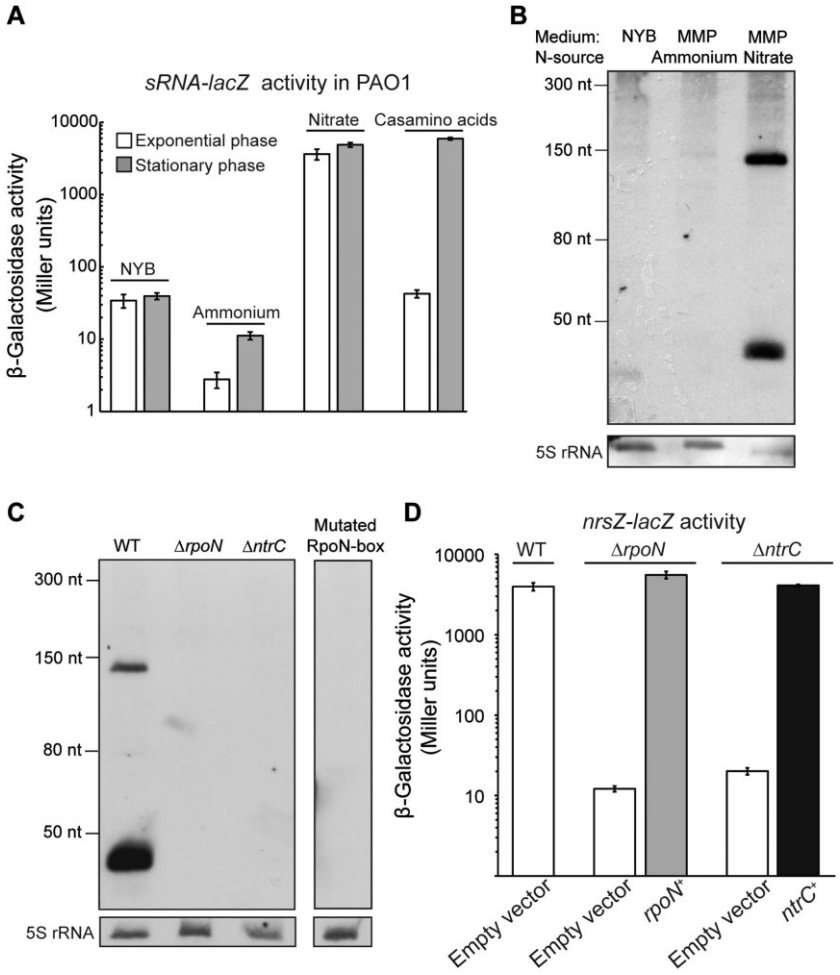

Fig. 1. The sRNA NrsZ of *P. aeruginosa* PAO1 is induced during nitrogen limitation by the NtrB/C-RpoN cascade.
A. β-Galactosidase activities of the chromosomal reporter fusion *sRNA-lacZ* (*nrsZ-lacZ*) under various nitrogen-limited conditions. The PAO1 WT strain carrying *nrsZ-lacZ* (PAO6750) was grown in NYB, MMP supplemented with glucose as carbon source and ammonia, nitrate or casamino acids (0.1%) as nitrogen source. Activity of the *nrsZ-lacZ* chromosomal fusion was measured in exponential phase and when stationary phase was reached. Each value represents the average of triplicate cultures ± standard deviation.
B. Northern blot detection of the sRNA: RNA was isolated from PAO1 (WT) grown to stationary phase in NYB, MMP supplemented with succinate as carbon source and ammonium or nitrate as nitrogen source. 7.5 μg of cross-linked total RNA was hybridized with the ssRNA probe *NrsRNA*. As loading control, the membrane was re-probed with the *5SDNA*, which detects 5S rRNA.
C. Northern blot detection of the sRNA: Total RNA was extracted from strains PAO1 WT, Δ*rpoN* (PAO6358), Δ*ntrC* (PAO6764), and from the strain mutated in the RpoN box of the *sRNA* promoter (PAO6846) grown to stationary phase in MMP supplemented with glucose and casamino acids (0.1%). 5 μg of cross-linked total RNA was hybridized with the ssRNA probe *NrsRNA*. As loading control, the membranes were re-probed with the *5SDNA* detecting the 5S rRNA.
D. β-Galactosidase activities of the chromosomal reporter fusion *nrsZ-lacZ* in different strains. The WT (PAO6750), Δ*rpoN* (PAO6847) and Δ*ntrC* (PAO6842) strains carrying the pME6001 empty vector, the strain Δ*rpoN* complemented with *rpoN*$^{+}$ (pME6001::*rpoN*, pME10389) and the strain Δ*ntrC* complemented with *ntrC*$^{+}$ (pME6001::*ntrC*, pME10390) were grown in MMP supplemented with glucose and casamino acids (0.1%). *nrsZ-lacZ* activity was measured when stationary phase was reached. Each value represents the average of triplicate cultures ± standard deviation.

the predicted promoter was investigated during growth with different nitrogen sources (N-sources), taking into account that RpoN is a regulator of nitrogen metabolism. We detected very low activity of the fusion in the rich medium nutrient yeast broth (NYB) and in the minimal medium P (MMP) supplemented with ammonium as the sole N-source (Fig. 1A). By contrast, the high activity of the fusion was measured when nitrate or casamino acids were used as sole N-sources. Furthermore, we observed that RpoN promoter activity was growth-phase independent in minimal medium containing nitrate as nitrogen source, whereas activity increased with cell density in minimal medium supplemented with a low concentration of casamino acids as N-source (Fig. 1A). The latter may be explained by the fact that in this condition, growth in 0.1% casamino acids medium is limited by N-source

availability, which suggests that nitrogen depletion triggers activity of the promoter. To test this hypothesis, we measured the growth of the PAO1 WT strain and the activity of the *sRNA-lacZ* fusion in MMP medium supplemented with different concentration of casamino acids. As expected, an increase in casamino acids concentration led to an increase in PAO1 growth rate and to a decrease in the expression of the fusion (Fig. S2). These results reveal that transcriptional activity of the RpoN promoter is induced under nitrogen limitation when a poor N-source, such as nitrate, is present or when cells undergo nitrogen starvation, i.e. growing to stationary phase with a limited N-source, such as low concentration of casamino acids.

To detect the putative sRNA encoded downstream of the RpoN consensus sequence, Northern blot analysis was performed. Total RNA was extracted from PAO1 cultures grown to stationary phase in NYB or in MMP supplemented with different N-sources. Northern blot experiments were carried out using a single-stranded RNA (*NrsRNA*) probe of 242 nt complementary to the region encoding the putative sRNA. As expected, transcripts were only detected when PAO1 was grown in MMP supplemented with nitrate (Fig. 1B) or with a limited concentration of casamino acids as the sole N-source (Fig. 1C). Surprisingly, two major transcripts of around 40 nt and 140 nt were detected. To investigate if the RpoN promoter generated both transcripts, Northern blot analysis was performed on the PAO6846 strain (where the RpoN consensus sequence of the WT PAO1 was mutated, as indicated in bold T(**GG**→**AA**)CACAGCC CCT(**GC**→**TT**)A) grown under nitrogen-limited conditions. In this strain, no transcript was detected (Fig. 1C), demonstrating that both the 40 nt and the 140 nt RNA forms are transcribed from the same RpoN promoter.

Taken together, these results show that an sRNA is encoded in the *ntrC*-PA5126 intergenic region of PAO1, that its transcription is induced during nitrogen limitation, and that it is processed into two short transcripts of around 40 nt and 140 nt. Therefore, due to the nitrogen source-dependent expression of the sRNA, we named the sRNA 'NrsZ', an acronym for nitrogen-regulated sRNA.

NrsZ is induced during nitrogen limitation by the NtrB/C-RpoN cascade

As suggested above, transcription of NrsZ is activated by the alternative sigma factor RpoN under nitrogen-limited conditions. Previous work demonstrated that the NtrB/C two-component system is activated under nitrogen-limited conditions and that nitrate used as a nitrogen source simulates the condition of nitrogen limitation (Kustu *et al.*, 1989; Rashedi *et al.*, 2005). As NtrB/C is known to activate transcription in concert with the alternative sigma factor RpoN, we reasoned that expression of NrsZ may also be regulated by NtrB/C.

To confirm that the transcription of NrsZ was NtrB/C- and RpoN-dependent, Northern blot analysis was performed on total RNA extracted from cultures of PAO1 (WT), *ΔrpoN* (PAO6358) and *ΔntrC* (PAO6764) mutant strains grown to stationary phase in MMP supplemented with glucose and casamino acids. Glucose was used as the carbon source to allow growth of the *ΔrpoN* mutant and casamino acids as a limited nitrogen source to allow growth of the *ΔntrC* mutant. The abundant 40 nt and 140 nt NrsZ transcripts observed in the WT strain were absent in both mutant strains (Fig. 1C), indicating that expression of NrsZ is RpoN- and NtrB/C-dependent. Moreover, as expected, NrsZ promoter activity was dramatically reduced in the Δ*ntrC* (PAO6842) and Δ*rpoN* (PAO6847) strains carrying the chromosomal *nrsZ-lacZ* reporter fusion (Fig. 1D). Complementation of the Δ*rpoN* and Δ*ntrC* mutant strains with the plasmid pME10389 or pME10390, carrying respectively the *rpoN* and *ntrC* genes under the control of the *lac* promoter, restored the expression of the *nrsZ-lacZ* reporter fusion to the WT level (Fig. 1D).

These results demonstrate that NrsZ is induced during nitrogen limitation by the NtrB/C two-component system in concert with RpoN. It is noteworthy that NrsZ is the first regulatory sRNA discovered to be activated by the NtrB/C-RpoN cascade in *P. aeruginosa* PAO1 in reaction to nitrogen starvation.

NrsZ is processed in three similar small forms

The observation that two transcripts of around 40 nt and 140 nt were generated from the same promoter led us to investigate the 3′ and 5′ ends of the respective RNA transcripts. For this purpose, we analyzed RNA deep sequencing data of directional full-length RNA from two fractionated small RNA samples. Briefly, RNA sequencing was performed on total RNA separated into two fractions, ranging from 30 nt to 200 nt (small fraction) and from 150 nt to 450 nt (medium fraction). One thousand four hundred eighty-one reads were mapped onto the *nrsZ* encoding strand located in the intergenic region *ntrC*-PA5126 (455 reads in the small fraction and 1026 reads in the medium fraction) (Fig. 2A and B), and covered all potential RNA molecules transcribed and derived from the *nrsZ* genomic position.

As observed in Fig. 2A and B, RNA size selection allowed the distinction of multiple processed forms of NrsZ. Although NrsZ is produced as a transcript of at least a 226 nt, only few molecules covered this entire sequence, indicating efficient maturation processes. In addition, abundant transcripts of around 40 nt and 130 nt long were detected. To better understand NrsZ

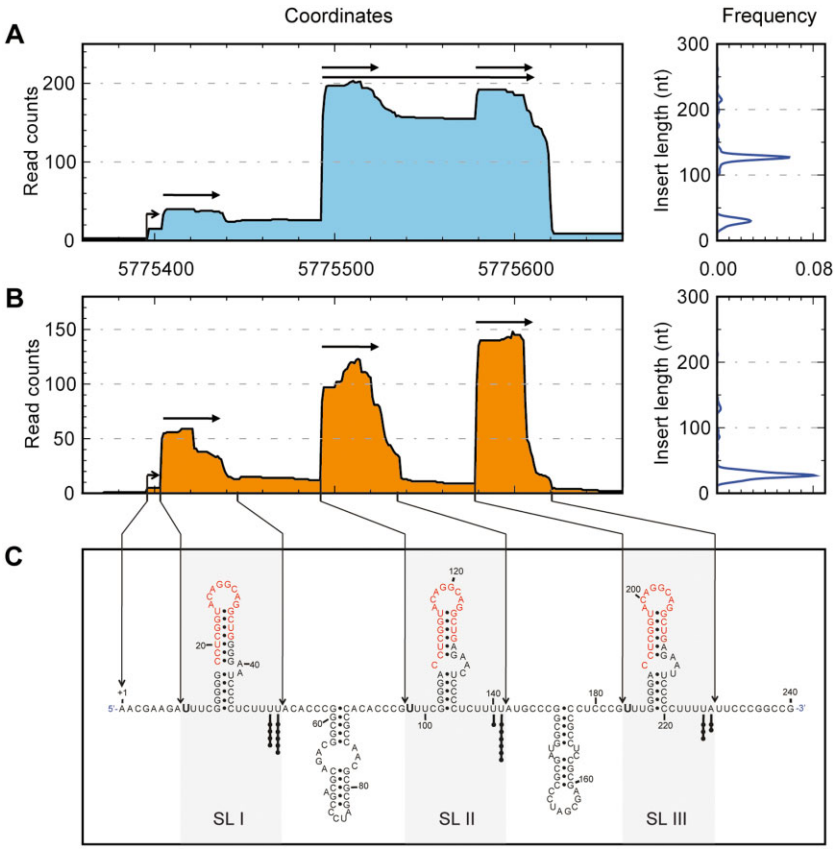

Fig. 2. NrsZ is processed in three small forms. The RNA deep sequencing profile of NrsZ was determined from RNA extracted from PAO1 grown in minimum medium supplemented with nitrate and fractioned in two parts, (A, left panel) the medium fraction ranging from 150 nt to 450 nt, and (B, left panel) the small fraction ranging from 30 nt to 200 nt. (A and B, right panels): insert length distributions of the transcripts corresponding to NrsZ in both fractions. Base numbering starts from the +1 transcription start site represented by a bent arrow, and horizontal arrows underline the major transcripts positions and sizes. (C) Predicted secondary structure of the NrsZ primary transcript. Each black dot represents one 3′ extremity of NrsZ obtained by 3′ RACE experiment. 5′-matured terminal nucleotides determined by RNA deep sequencing are indicated in bold. Conserved motifs in the stem-loop sequences are shown in red. SL: stem-loop structure. The MFOLD program was used to predict RNA secondary structures (Zuker, 2003).

processing, NrsZ secondary structure analysis was performed using the MFOLD program (Zuker, 2003) (Fig. 2C). NrsZ was predicted to form five stem-loop structures (SL), of which three present conserved residues in the loop (SL I–III). Analysis of the RNA sequencing in the medium fraction showed that two major forms of NrsZ were prevalent (Fig. 2A): short forms of around 44 nt composed by the SL I, II and III (starting at residue 9 and ending at residue 52, 98 to 142 and 184 to 226), and a long form of 127 nt composed of the SL II and SL III RNA (starting from residue 98 and ending at residue 226; Fig. 2A and 2C). These two major transcripts matched with the two major bands detected by Northern blot experiments (Fig. 1B and C). RNA sequencing results of the small fraction demonstrated that NrsZ was also detected as three abundant small forms of around 44, 43 and 41 nt, corresponding to the sequences of the three individual SL I–II–III respectively. Interestingly, these three forms were present in a relatively equal amount. Surprisingly, the RNA sequencing mapping indicated that only few transcripts started at the predicted transcription start site located 12 nt downstream of the RpoN consensus sequence, and that the 5′ and 3′ extremities of the transcripts enclosed three and four U residues respectively (Fig. 2C). To confirm the RNA deep sequencing results, 3′ and 5′ RACE (rapid amplification of cDNA ends) experiments were performed. Twenty-one independent sequences were obtained from the 3′RACE experiments, and were mapped to the U_{51}-U_{52}, U_{142}-U_{141} and U_{225}-A_{226} residues, as presented in Fig. 2C. The 3′RACE results were in agreement with those obtained from RNA sequencing that the terminal 3′ extremities were located after each SL I–III (Fig. 2C). Furthermore, it is noteworthy that the 127 nt long transcript detected in the medium fraction, encompassing SL II and SL III, is also processed into two shorter fragments of 43 nt and 41 nt that are detected in the small fraction and encompass SL II and SL III respectively. 5′ RACE analysis was also attempted, but technical problems did not permit the identification of the 5′ extremities of NrsZ (data not shown).

NrsZ regulates P. aeruginosa *swarming motility*

To identify the biological function(s) of NrsZ in *P. aeruginosa* PAO1, we used a phenotypic approach. We demonstrated that NrsZ expression is NtrB/C-dependent, and is therefore induced under nitrogen-limited conditions. It was previously demonstrated that nitrogen-limited conditions are necessary to sustain *P. aeruginosa* swarming motility (Köhler *et al.*, 2000). Consequently, we tested the swarming motility of *P. aeruginosa* PAO1 (WT) and PAO1 NrsZ negative strain (PAO6846, *nrsZ*$^{-}$) carrying the

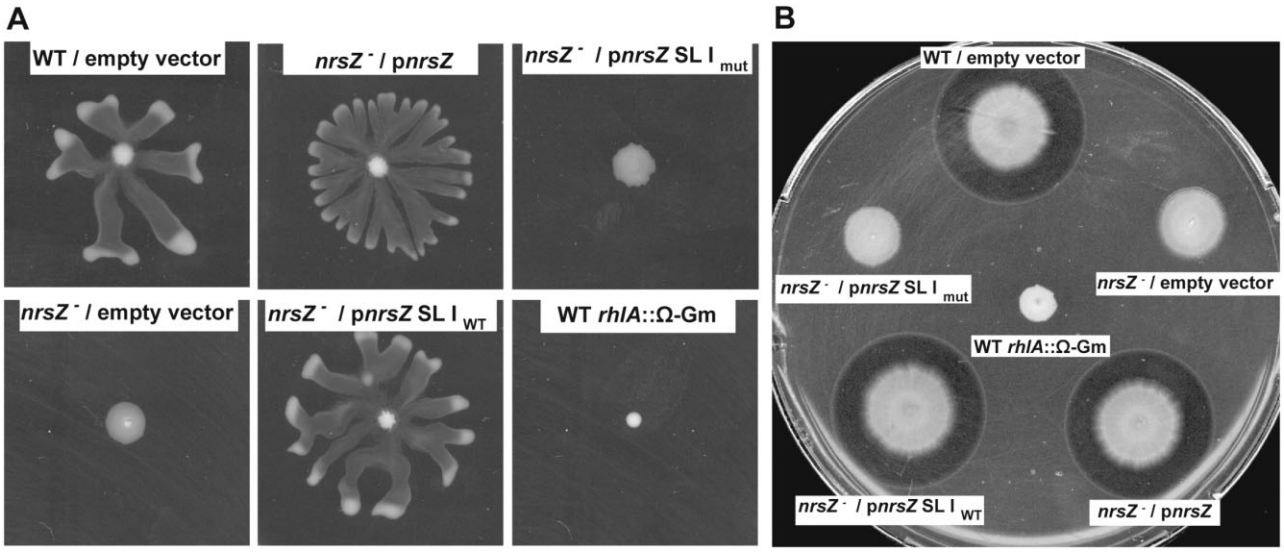

Fig. 3. The *P. aeruginosa nrsZ*⁻ mutant is defective for swarming motility and rhamnolipids production. Swarming motility (A) and rhamnolipids production (B) were assayed as described in the *Experimental procedures* for strains PAO1 WT and PAO1 *nrsZ*⁻ (PAO6846) containing the empty vector pME4510 or its derivative plasmids pME9995, pME10138 and pME10191, encoding full length *nrsZ* (p*nrsZ*), truncated *nrsZ* (p*nrsZ* SL I_{WT}) or the mutated truncated *nrsZ* (p*nrsZ* SL I_{mut}) respectively. As control, motility- and rhamnolipid-defective mutant PT712 (PAO1*rhlA*::Ω-Gm) was tested in the same condition. All experiments were performed in four replicates. The pictures depict a representative plate for each experiment.

empty plasmid pME4510. The mutant strain showed a complete swarming defect that could be restored by introducing the WT *nsrZ* gene under control of its native promoter (pME9995, Fig. 3A). Interestingly, the 60 first nucleotides of the *nrsZ* gene encoding the SLI (pME10138) were also able to revert the swarming-defective phenotype of the PAO1 *nrsZ*⁻ mutant, indicating that the first 60 nt of NrsZ is sufficient to regulate swarming motility in *P. aeruginos*a PAO1 (Fig. 3A). Twitching and swimming motilities of the *nsrZ* mutant strain were also tested, but no modulation of these phenotypes was observed (data not shown). These results show that NrsZ is a regulator of *P. aeruginosa* swarming motility and that the first 60 nt of NrsZ containing the SLI acts as a functional unit sufficient for swarming regulation.

NrsZ regulates rhamnolipids production

In order to swarm, *P. aeruginosa* secretes rhamnolipid biosurfactants (Caiazza *et al*., 2005). Furthermore, rhamnolipids production was observed to be enhanced in the presence of nitrate in comparison to ammonium as nitrogen source (Guerra-Santos *et al*., 1984; Lang and Wullbrandt, 1999; Maier and Soberón-Chávez, 2000). As is the case in rhamnolipids production, NrsZ is induced during nitrogen limitation, and as we showed positively regulates swarming motility. These considerations led us to investigate the involvement of NrsZ in rhamnolipids production by *P. aeruginosa* PAO1. We tested the production of rhamnolipids on plates containing the same nutrient composition used for swarming assays. In comparison to PAO1 WT, PAO1 *nrsZ*⁻ carrying the empty plasmid pME4510 was clearly deficient for rhamnolipids production (Fig. 3B). The complementing plasmids pME9995 (carrying *nrsZ*) or pME10138 (carrying the first 60 nucleotides of *nrsZ*) fully restored rhamnolipids production of the PAO1 *nrsZ*⁻ mutant. These results indicate that NrsZ, and in particular its first 60 nt, positively regulates rhamnolipids production.

NrsZ activates rhlA expression at the post-transcriptional level

Synthesis of the rhamnolipid biosurfactants in *P. aeruginosa* is mediated by a biosynthetic pathway involving the *rhlAB* gene cluster (Maier and Soberón-Chávez, 2000; Soberón-Chávez *et al*., 2005). Importantly, an *rhlA* mutant is defective in rhamnolipids production and swarming motility (Köhler *et al*., 2000; Déziel *et al*., 2003). As NrsZ regulates rhamnolipids production, we investigated whether NrsZ regulates *rhlA* expression. Expression of the translational *rhlA'-'lacZ* reporter fusion (pECP60) was first measured in the PAO1 WT strain grown in medium containing the same nutrient composition used for swarming motility and rhamnolipids analyses. In these conditions, *rhlA* expression increased with increasing cell density to reach a very high expression level ($40{\cdot}10^3$ Miller units). We then measured

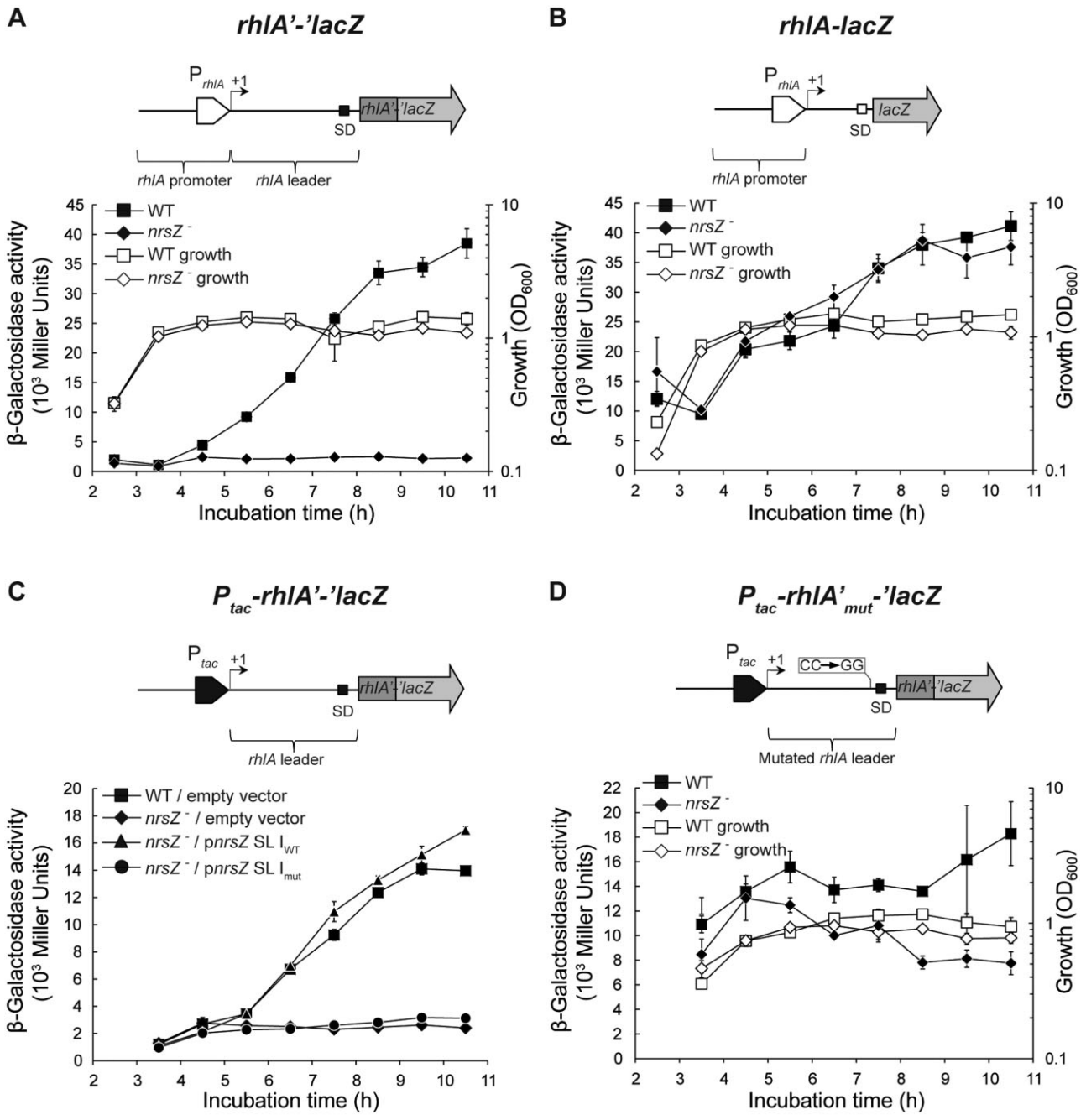

Fig. 4. *RhlA* expression is activated by NrsZ at the post-transcriptional level. Cell density dependent β-galactosidase activities of (A) the translational *rhlA'-'lacZ* reporter fusion (pECP60) and of (B) the transcriptional *rhlA-lacZ* fusion (pME3838) in PAO1 WT and PAO1 *nrsZ*⁻ (PAO6846). (C) Expression of the translational P_{tac}-*rhlA'-'lacZ* fusion (pME10173) in PAO1 WT and PAO1 *nrsZ*⁻, containing the empty vector pME4510 or its derivative plasmids pME10138 and pME10191, which encode for truncated *nrsZ* (p*nrsZ* SL I_{WT}) and mutated, truncated *nrsZ* (p*nrsZ* SL I_{mut}) respectively. (D) β-galactosidase activities of the translational P_{tac}-*rhlA*$_{mut}$*'-'lacZ* (pME10182) fusion in PAO1 WT and PAO1 *nrsZ*⁻. Cultures were grown in MMP supplemented with glucose and casamino acids (0.1 %); growth curves are represented with the indicated symbols. Each value represents the average of three cultures ± standard deviation. Schematics for each fusion are represented: the promoters, the transcription start sites (+1), the Shine-Dalgarno sequences (SD) and the mutations (in a box) are indicated.

β-galactosidase activity of the *rhlA'-'lacZ* fusion in the PAO1 *nrsZ*⁻ mutant strain and observed that the expression of *rhlA* was completely repressed in the mutant strain in comparison with the WT strain, indicating that NrsZ positively regulates *rhlA* expression (Fig. 4A). By contrast, no difference of the transcriptional *rhlA-lacZ* fusion (pME3838) activity was detected between both strains (Fig. 4B). The expression of the P_{tac}-*rhlA'-'lacZ* fusion

(pME10173), carrying the constitutive strong *tac* promoter, was increasing following growth in the WT strain (Fig. 4C), similarly to the *nrsZ* expression profile observed in the same condition (Fig. S2). However, the P_{tac}-*rhlA'*-'*lacZ* fusion activity was subject to a strong repression in PAO1 *nrsZ*$^-$ and stayed at a basal level during all cell growth (Fig. 4C). In addition, the complementing plasmid pME10138, carrying the first 60 nt of NrsZ fully restored *rhlA* expression in PAO1 *nrsZ*$^-$ (Fig. 4C).

Taken together, these results showed that NrsZ regulates positively *rhlA* at the post-transcriptional level and confirmed that NrsZ short transcript is a sufficient functional unit for the activation of *rhlA* expression.

The conserved motif ACAGGCAG in the loop of NrsZ is essential for rhlA activation

The predicted secondary structure of NrsZ suggests the presence of three repeated SL with a conserved ACAGGCAG motif in their loop (Fig. 2C). Therefore, we hypothesize that this sequence is involved in the activation of *rhlA* expression. To test this hypothesis, we constructed the plasmid pME10191, carrying the first 60 nt of NrsZ mutated in the ACA(**GG**→**CC**)CAG motif and transformed the plasmid into PAO6846. The plasmid did not restore *rhlA* expression in PAO1 *nrsZ*$^-$ (Fig. 4C), and neither swarming motility nor rhamnolipids production of this strain was recovered (Figs 3A and B, and 4C). These results indicate that the ACA**GG**CAG stem-loop motif of NrsZ is crucial for NrsZ regulatory function.

Next, to identify the binding site of NrsZ on *rhlA* mRNA, we hypothesized that the ACAGGCAG motif of NrsZ is exposed and involved in NrsZ-*rhlA* RNA duplex formation. We, therefore, screened the leader sequence of *rhlA* mRNA for a potential complementary sequence to this motif. Our analysis predicted that the NrsZ stem-loop motif interacts with a region present upstream of the *rhlA* ribosome binding site (Fig. 5). We then constructed a P_{tac}-*rhlA'*-'*lacZ* fusion mutated in the NrsZ binding site GUUUG(**CC**→**GG**)UGUUCGA (P_{tac}-$rhlA_{mut}$'-'*lacZ*; pME10182), and tested its expression in PAO1 WT and PAO1 *nrsZ*$^-$. In exponential growth phase, the expression of the P_{tac}-$rhlA_{mut}$'-'*lacZ* fusion was clearly de-repressed in the WT strain compared with the P_{tac}-*rhlA'*-'*lacZ* fusion in the same condition (Fig. 4C and D). In addition, the expression of the P_{tac}-$rhlA_{mut}$'-'*lacZ* fusion was similar in both the WT and *nrsZ*$^-$ mutant. However, a slight increase of the P_{tac}-$rhlA_{mut}$'-'*lacZ* fusion activity was observed in stationary growth phase in the WT strain compared with the mutant strain (Fig. 4D), suggesting that this mutation is not sufficient to impair NrsZ regulation. These results suggest a role for this region in the regulation of *rhlA* expression and in NrsZ-mediated regulation.

Discussion

NrsZ is, until now, the only described sRNA regulated by the NtrB/C system and shown to positively modulate *P. aeruginosa* swarming motility. Our study shows that during nitrogen limitation, NrsZ modulates swarming motility of *P. aeruginosa* by activating *rhlA* expression, leading to an increase in the production of rhamnolipids, important virulence factors of *P. aeruginosa* (Figs 3A and B, and 4). Previous studies already demonstrated that *rhlA* expression and rhamnolipids production are dependent on nitrogen availability in *P. aeruginosa* (Mulligan and Gibbs, 1989; Ochsner *et al*., 1994) and on the alternative sigma factor RpoN (Pearson *et al*., 1997). However, the mechanisms leading to these regulations were not fully elucidated. Nevertheless, it has been suggested, using translational reporter fusions, that RpoN activates *rhlA* expression indirectly through the activation of the transcription of *rhlR* (encoding an *rhlAB* transcriptional activator) (Medina *et al*., 2003a,b). Based on our results, however, we propose that activation of *rhlA* expression by nitrogen limitation and RpoN is indirect and post-transcriptional due to the regulation of *rhlA* expression at the post-transcriptional level by NrsZ, an sRNA induced by the NtrB/C-RpoN cascade.

To obtain more insight into NrsZ regulatory function, MFOLD analysis of NrsZ secondary structure was performed and predicted the formation of three highly conserved stem-loop structures separated by two stem-loop spacers (Fig. 2C). Furthermore, our study demonstrated that NrsZ is processed into two transcripts of around 40 nt and 140 nt, and that NrsZ regulatory activity can be mediated solely by the short abundant processed fragment encompassing the first predicted stem-loop structure (SL I), which exposes the conserved RNA sequence motif ACAGGCAG in the loop. These results would support the theory that NrsZ belongs to a category of sRNAs, such as ArcZ and MicX, that require processing after transcription to produce active regulatory molecules (Davis and Waldor, 2007; Papenfort *et al*., 2009). Our results suggest that specific and efficient endoribonucleolytic cleavage of the 5′ end and 3′ exonucleolytic trimming of NrsZ occur, and we hypothesize that these mechanisms are triggered by RNAseE or RNAse G, and PNPase, RNAseR or RNase II respectively. It is noteworthy that the two spacers, positioned between SL I–SL II and SL II–SL III, and having the same size and structure but different sequences, seem unstable and are quickly degraded by ribonucleases. This leads to the production of short and efficient matured regulatory NrsZ transcripts. Intriguingly, the predicted NrsZ structure resembles that of the CRISPR system that is composed of multiple structured short direct repeats separated by spacers. However, as revealed by a recent study, *P. aeruginosa* strain PAO1

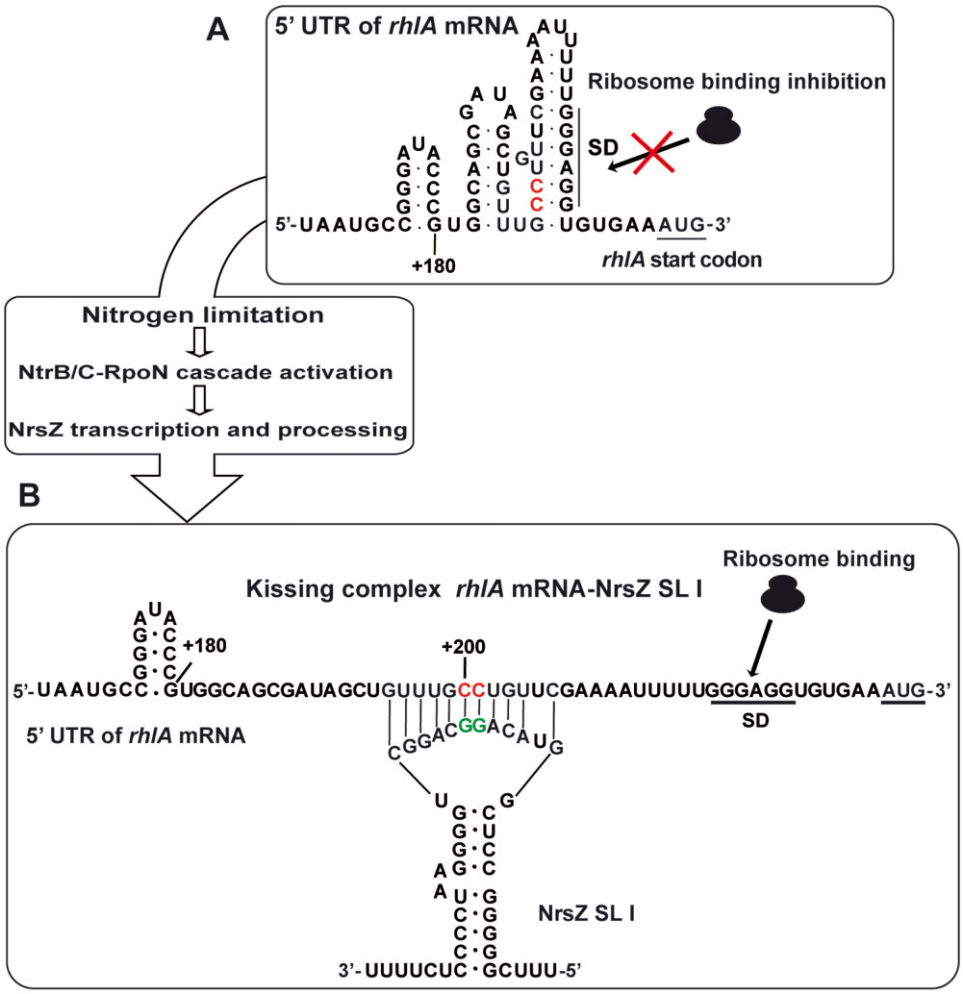

Fig. 5. Model of the *rhlA*-NrsZ kissing complex formation that leads to the activation of *rhlA* mRNA translation.
A. In the absence of NrsZ, *rhlA* translation is repressed by a self-inhibitory secondary structure on the 5' untranslated region (5' UTR) of its mRNA. This structure sequesters the *rhlA* Shine-Dalgarno sequence (SD), and therefore ribosome binding, leading to the inhibition of *rhlA* mRNA translation.
B. Under nitrogen limitation, NrsZ is induced by the NtrB/C-RpoN cascade and is processed in short, functional stem-loop molecules (NrsZ SL I, II, III). These molecules base-pair with their identical loop sequence (GUACAGGCAGGC) with the *rhlA* 5' UTR forming a kissing complex. This base-pairing allows melting of the self-inhibitory structure of *rhlA*, liberating the SD for ribosome binding. Therefore, *rhlA* mRNA translation is activated, triggering rhamnolipids production and swarming motility. For simplification, only the NrsZ SL I molecule is presented. Nucleotides mutated (CC→GG) in the fusion P_{tac}-*rhlA*$_{mut}$'-'*lacZ* (pME10182) are indicated in red. Nucleotides mutated (GG→CC) in the plasmid expressing NrsZ SL I$_{mut}$ (pME10191) are indicated in green. The MFOLD program was used to predict RNA secondary structures (Zuker, 2003).

lacks a CRISPR/Cas system (Ferrara *et al.*, 2012). To better characterize NrsZ function, the roles played by all three highly conserved stem-loop structures are currently being investigated.

One challenge in studying novel sRNAs is to decipher their mode of action. The sRNAs characterized so far and involved in post-transcriptional regulation exert their regulatory functions using two main mechanisms: capture of small RNA-binding proteins of the RsmA/CsrA type or base-pairing with an mRNA target. To test the hypothesis that NrsZ titrates an RNA-binding protein, we carried out a transposon mutagenesis in the *P. aeruginosa* PAO1 *nrsZ*$^-$ strain containing the translational *rhlA*'-'*gfp* fusion. In this condition, *rhlA* expression is impaired due to repression by the RNA-binding protein. If the gene coding for this RNA-binding protein repressor is mutated, *rhlA* expression will be restored. Unfortunately, we did not identify any putative protein targets despite having carried out an extensive screening (data not shown), suggesting that either the putative protein is essential or that NrsZ regulation does not involve a protein partner.

To activate translation initiation, sRNAs have been generally shown to bind to the 5′ region of their mRNA targets (Waters and Storz, 2009). In line with this theory, we

predicted that the NrsZ stem-loop motif could interact with a region upstream the *rhlA* Shine-Dalgarno (SD) by forming a kissing complex. Using the secondary structure prediction MFOLD program, we observed that the *rhlA* leader mRNA seems to be well structured and that a stem-loop structure might engulf the SD, conceivably leading to an inhibition of translation. Upon binding of NrsZ, this stem-loop structure would dissociate and liberate the *rhlA* SD for initiation of translation (Fig. 5). To validate the predicted interaction, we performed electrophoretic mobility shift assays, as previously described (Møller *et al*., 2002; Lease and Woodson, 2004), using the short transcript form of NrsZ and the *rhlA* leader mRNA. However, no binding was detected (data not shown). We also carried out mutational analyses. Expression of the *rhlA* reporter fusion mutated in the putative NrsZ binding site was dramatically less subjected to NrsZ regulation, indicating that the two mutated residues are probably part of the *rhlA* mRNA-NrsZ recognition and binding site (Fig. 4D). However, we observed that the level of expression of the P_{tac}-*rhlA*$_{mut}$'-'*lacZ* fusion was similar to that of the P_{tac}-*rhlA*'-'*lacZ* fusion in late stationary phase (Fig. 4D). This result could be explained by the fact that the mutated residues are involved in stem formation of the stem-loop structure engulfing the SD, and therefore breakage of this stem (a result of the sequence mutation) would lead to liberation of the *rhlA* SD and increase of its expression. To counteract this undesirable regulation, we envisaged construction of a new P_{tac}-*rhlA*$_{mut}$'-'*lacZ* fusion carrying compensatory mutations to preserve integrity of the stem-loop structure. However, compensatory mutations in this region would alter the SD sequence, leading to dramatic effects on the expression of the reporter fusions. Therefore, the NrsZ mechanism of action is still unknown and is currently under investigation.

To emphasize the global role of NrsZ in *Pseudomonas*, we predict that the NrsZ stem-loop structures with an ACAGGCAG motif in their loop are highly conserved and widely distributed among Pseudomonads, independently of their ecological features (Fig. S3A and B). In addition, a conserved RpoN promoter is predicted to control transcription of the different *nrsZ* species. Interestingly, the copy number of the NrsZ conserved stem-loop sequence and structure is variable among different *Pseudomonas* strains. For example, the secondary structure of *P. protegens* Pf-5, *P. putida* KT2442, *P. entomophila* L48 and *P. syringae* pv. tomato DC3000 NrsZ is predicted to form six, three, two and only one conserved stem-loop structures separated by stem-loop spacer respectively (Fig. S3B). Interestingly, the length of the spacers (from 3′-U triplet to 5′-U triplet) in each *Pseudomonas* specie is well conserved, and only small variations in length are observed for the last spacer sequence, while the length of the spacers in between species is variable (Fig. S3B). This observation raises numerous questions on the acquisition and divergence of NrsZ sequence in Pseudomonads.

It is noteworthy that complementation experiments showed that when the PAO1 *nrsZ*$^-$ mutant strain, deficient for rhamnolipids production, was complemented with plasmids carrying either *P. protegens* Pf5, *P. putida* KT2442, *P. entomophila* L48 or *P. syringae* pv. tomato DC3000 NrsZ under the control of their native promoter, rhamnolipids production was restored (Fig. S4). These results demonstrate that NrsZ can be exchanged and functional in the different Pseudomonads strains. Similarly, swarming motility impaired in the mutant strain was restored by complementation experiments (data not shown).

It is well known that in addition to its capacity to colonize eukaryotic hosts like other *Pseudomonas* species, *P. aeruginosa* is widely distributed in soil as well as in fresh or sea water (Green *et al*., 1974; Hardalo and Edberg, 1997; Kimata *et al*., 2004). In most of these niches, nitrogen is probably limited. Therefore, we hypothesize that the regulatory sRNA NrsZ is an important regulator of *P. aeruginosa* virulence and is crucial for the adaptation of Pseudomonads in these oligotrophic conditions, ensuring an optimal physiological response to thwart nitrogen starvation.

Experimental procedures

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 1 and the oligonucleotide sequences in Table S1. Cells were grown at 37°C in the NYB medium, in Luria-Bertani broth medium (LB) or in minimal medium P (MMP) (Haas *et al*., 1977) supplemented with 20 mM succinate, mannitol or glucose as carbon source, and 20 mM ammonium (NH_4Cl), 20 mM nitrate (KNO_3) or 0.1% (w/v) casamino acids as nitrogen source. When required, antibiotics for *P. aeruginosa* were used at the following concentrations: 2000 μg ml^{-1} carbenicillin, 25 μg ml^{-1} gentamicin and 125 μg ml^{-1} tetracycline, and 100 μg ml^{-1} ampicillin, 10 μg ml^{-1} chloramphenicol, 25 μg ml^{-1} kanamycin, 10 μg ml^{-1} gentamicin and 25 μg ml^{-1} tetracycline for *E. coli*.

Construction of plasmids and strains

The intergenic region *ntrC*-PA5126 containing *nrsZ* and the sequence containing the promoter to the 60th nucleotide after the predicted start of transcription of *nrsZ* were amplified using chromosomal DNA of *P. aeruginosa* PAO1, and primer pairs 2913FW/2913REV and NRS-HP1-REV/sRNA2913FW2 respectively. The obtained Polymerase Chain Reaction (PCR) products of 1.015 and 0.4 kb were digested with BamHI and HindIII, and ligated into the corresponding sites in pME4510, resulting in pME9995 and pME10138 respectively. pME10191 was constructed by ligating a PCR fragment of 0.4 kb, amplified using primers

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Description[a]	References
Strains		
Pseudomonas		
P. aeruginosa		
PAO1	Wild-type	Holloway and colleagues (1979)
PAO6358	PAO1 Δ*rpoN*	Heurlier and colleagues (2003)
PAO6750	PAO1, *nrsZ-lacZ*; Tc^r	This study
PAO6764	PAO1 Δ*ntrC* carrying a 1.41 Kb in-frame deletion in *ntrC*	This study
PAO6842	PAO6764, *nrsZ-lacZ*; Tc^r	This study
PAO6846	PAO1 *nrsZ*$^-$, mutation in the RpoN-box of the *nrsZ* promoter	This study
PAO6847	PAO6358, *nrsZ-lacZ*; Tc^r	This study
P. protegens		
Pf-5	Wild-type	Paulsen and colleagues (2005)
P. putida		
KT2442	Spontaneous rifampicin-resistant mutant of the KT2440 wild-type strain	Franklin and colleagues (1981), and Nelson and colleagues (2002)
P. entomophila		
L48	Wild-type	Vodovar and colleagues (2006)
P. syringae		
pv. tomato DC3000	Wild-type	Buell and colleagues (2003)
E. coli		
DH5α	*recA1, endA1, hsdR17, deoR, thi-1, supE44, gyrA96, relA1,* Δ(*lacZYA-argF*), *U169*(φ80d*lacZ*ΔM15)	Sambrook and colleagues (2001)
HB101	*hsdS, recA, proA2, leu-6, ara-14, galK2, lacY1, xyl-5, mtl-1, rpsL20, thi-1, supE44*	Sambrook and colleagues (2001)
Plasmids		
pECP60	*rhlA'-'lacZ* fusion; Ap^r, Cb^r	Pesci and colleagues (1997)
pGEM-T easy	Cloning vector; Ap^r	Promega
pUX-BF13	mini-Tn*7* transposition helper plasmid; Ap^r	Bao and colleagues (1991)
pME497	Mobilizing plasmid, IncP-1, Tra RepA (Ts); Ap^r	Voisard and colleagues (1994)
pME3087	Suicide plasmid, Co1E1 replicon; Tc^r	Voisard and colleagues (1994)
pME3838	*rhlA-lacZ* fusion; Tc^r	Heurlier and colleagues (2004)
pME4510	Multicopy broad host range plasmid; Gm^r	Rist and Kertesz (1998)
pME6001	Cloning vector; Gm^r	Blumer and colleagues (1999)
pME6015	Translational *lacZ* fusion cloning vector; Tc^r	Schnider-Keel and colleagues (2000)
pME6016	Transcriptional *lacZ* fusion cloning vector; Tc^r	Schnider-Keel and colleagues (2000)
pME6552	pUK21::P_{tac}; Km^r	Blumer and colleagues (1999)
pME7549	Mini-Tn*7*-Tc delivery plasmid; Tc^r, Ap^r	C. Reimmann (unpublished)
pME9989	pME6016 carrying a 0.314 kb insert of the *nrsZ* promoter (*nrsZ-lacZ*); Tc^r	This study
pME9991	Suicide plasmid for deletion of *ntrC*; Tc^r	This study
pME9995	pME4510 derivative carrying a 1.015 kb insert encompassing *nrsZ*; Gm^r	This study
pME10129	pME7549 derivative carrying the *nrsZ-lacZ* fusion; Tc^r, Ap^r	This study
pME10134	Suicide plasmid for mutation of the RpoN-box of the *nrsZ* promoter; Tc^r	This study
pME10138	pME4510 derivative carrying a 0.4 kb insert containing the first 60 nucleotides of *nrsZ*; Gm^r	This study
pME10142	pME4510 derivative carrying a 0.797 kb insert encompassing *nrsZ* of *P. protegens* Pf-5; Gm^r	This study
pME10143	pME4510 derivative carrying a 0.563 kb insert encompassing *nrsZ* of *P. putida* KT2242; Gm^r	This study
pME10144	pME4510 derivative carrying a 0.425 kb insert encompassing *nrsZ* of *P. entomophila* L48; Gm^r	This study
pME10145	pME4510 derivative carrying a 0.408 kb insert encompassing *nrsZ* of *P. syringae* pv. tomato DC3000; Gm^r	This study
pME10171	pME6015 derivative carrying the P_{tac} promoter; Tc^r	This study
pME10173	pME10171 derivative carrying the P_{tac} –*rhlA'-'lacZ* fusion; Tc^r	This study
pME10182	pME10173 derivative with $CC_{(200–201)} \rightarrow GG_{(200–201)}$[b] mutations, P_{tac} –*rhlA*$_{mut}$*'-'lacZ* fusion; Tc^r	This study
pME10191	pME10138 derivative with $GG_{(28–29)} \rightarrow CC_{(28–29)}$[b] mutations; Gm^r	This study
pME10389	pME6001 derivative carrying *rpoN* in a 1.579 kb insert; Gm^r	This study
pME10390	pME6001 derivative carrying *ntrC* in a 1.552 kb insert; Gm^r	This study

a. Antibiotic resistance phenotypes are indicated by ^{r}: Ap, ampicillin, Gm, gentamicin; Tc, tetracycline; Km, kanamycin; Cb, carbenicillin.
b. Nucleotide numbers correspond to the +1 transcription starts nucleotides.

sRNA2913FW2 and NrsZRevmodifIV, and digested with BamHI and HindIII, into pME4510 digested with the same restriction enzymes.

The intergenic region encoding for the homologs of *nrsZ* in *P. protegens* Pf-5, *P. putida* KT2442, *P. entomophila* L48 and *P. syringae* pv. tomato DC3000 was amplified using chromosomal DNA of each strains, and primer pairs Pf5-2913FW/Pf5-2913REV, KT2442-2913FW/KT2442-2913REV, L48-2913FW/L48-2913REV and DC3000-2913FW/DC3000-2913REV respectively. For the three first strains (Pf-5, KT2442, L48), the corresponding 797 bp, 563 bp and 425 bp PCR products were digested with EcoRI and BamHI, and ligated in the corresponding site of pME4510, resulting in plasmid pME10142, pME10143 and pME10144 respectively. For the fourth strain (DC3000), the 408 bp PCR product was digested with BamHI and HindIII, and ligated into the corresponding site of pME4510, resulting in plasmid pME10145.

To construct the *nrsZ* transcriptional reporter fusions, the promoter region of *nrsZ* was amplified by PCR using primers sRNA2913FW2 and sRNA2913REV2 with pME9995 as DNA template. The PCR fragment of 314 bp was digested with EcoRI and BamHI, and ligated into pME6016 digested with the same enzymes, resulting in pME9989 (*nrsZ-lacZ*). The *nrsZ* promoter fused to *lacZ* was excised from pME9989 by digestion with BamHI and XhoI, and the 3.5 kb fragment was blunted and ligated into pME7549 digested with StuI, resulting in pME10129.

To construct pME10173 carrying the P_{tac}-*rhlA*'-'*lacZ* reporter fusion, a fragment of 300 bp containing the sequence from the start of transcription [previously determined (Pearson *et al*., 1997)] to the 24th codon of the *rhlA* gene was amplified by PCR using the primer pair RhlA-FW-K/RhlA-REV-P. The fragment was digested with KpnI and PstI, and cloned into the corresponding sites in pME10171.

Plasmid pME10171 was constructed from ligation of the P_{tac} promoter (0.9 kb) excised from pME6552 with StuI and EcoRI, and a 2 kb fragment of pME6015 digested with EcoRI and SacI into pME6015 digested with HindIII blunted and SacI. The plasmid pME10182 carrying the P_{tac}-*rhlA*$_{mut}$'-'*lacZ* mutant reporter fusion was constructed as follows: a 217 bp and 129 bp fragments were amplified by PCR from pME10173 using primer pairs RhlA-FW-K/RhlA-MutIIIA and RhlA-FW-P/RhlA-MutIIIB respectively. The two fragments were fused and amplified by overlap extension PCR (Heckman and Pease, 2007) using primers RhlA-FW-K and RhlA-FW-P, resulting in a 320 bp fragment that was digested with KpnI and PstI, and ligated into the corresponding sites in pME10171.

The *nrsZ-lacZ* transcriptional fusion carried by the mini-Tn*7* delivery plasmid pME10129 was inserted into the Tn*7* attachment site of PAO1, PAO6764 and PAO6358, yielding strains PAO6750, PAO6842 and PAO6847, respectively, using the pUXBF-13 transposition helper plasmid (Bao *et al*., 1991).

Chromosomal inactivation of the *ntrC* gene in *P. aeruginosa* PAO1 was performed as follows: PCR fragments of 500–600 bp corresponding to the flanking regions of *ntrC* were amplified from *P. aeruginosa* genomic DNA with primer pairs ntrC1/ntrC2 and ntrC3/P33REV1. Fragments were fused together by overlap extension PCR, digested with BamHI and HindIII, and ligated into the corresponding sites of the suicide vector pME3087, resulting in pME9991. Triparental mating was performed to mobilize plasmid pME9991 from *E. coli* DH5α into *P. aeruginosa* PAO1 using the mobilizing plasmid pME497 carried by *E. coli* HB10, and merodiploids were resolved as described before (Ye *et al*., 1995). Strain PAO6764 carrying an in-frame *ΔntrC* mutation was generated.

For inactivation of the *nrsZ* gene, a mutation in the RpoN-box (T**AA**CACAGCCCC**TT**TA in place of T**GG**$_{-24}$CACAGCCCCT**GC**$_{-12}$A) of the *nrsZ* promoter region was created. Two 670 bp fragments were amplified by PCR using the primer pairs 2913FW/sRNA2913rpoN1 and sRNA2913rpoN2/sRNA2913del5. Fragments were fused by overlap extension PCR, digested with BamHI and HindIII, and ligated into the corresponding sites of the suicide vector pME3087. The resulting plasmid pME10134 was used as described above to construct PAO6846.

The plasmids pME10389 or pME10390 used for complementation experiments were obtained by cloning, respectively, the *rpoN* and *ntrC* genes in the pME6001 multi-copy plasmid under the *lac* promoter. *rpoN* and *ntrC* were amplified by PCR with primer pairs rpoN-FWcomp/rpoN-REVcomp or ntrC-FWcomp/ntrC-REVcomp, respectively, and cloned into pME6001 after digestion with EcoRI/BamHI.

Northern blot analysis

The total RNA was isolated from cells grown in different media (as described in figure legends) using the hot acid phenol method, as previously described (Leoni *et al*., 1996). RNA fractions were treated with DNase I recombinant (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. The 5S rRNA DIG-labelled, double-stranded DNA (dsDNA) probe (*5SDNA*) was synthesized, as described before (Sonnleitner *et al*., 2009), with the primer pair 5S-rRNA-1/5S-rRNA-2.

The anti-NrsZ DIG-labelled, single-stranded RNA (ssRNA) probe of 242 nt (*NrsRNA*), covering a region encompassing the predicted +1 nucleotide of NrsZ (12 nt after the G nt of the −12 box of the RpoN-box sequence) to the last 17 nt of the PA5126 gene, was synthesized with T7 RNA polymerase (Promega, Madison, WI, USA) from a PCR DNA template obtained using pME9995, and the primer pair Nrs_FW_P and T7-2913 probe, according to the manufacturer's recommendations (DIG RNA Labeling Mix, Roche Diagnostics). Prior to hybridization, dsDNA and ssRNA DIG-labelled probes were heat-denatured for 10 min at 100°C or 68°C respectively. Of the total RNA, 5′10 μg were loaded onto the denaturing urea-polyacrylamide gels and analyzed by Northern blotting, as previously described (González *et al*., 2008). Primer sequences are listed in Table S1.

Rapid amplification of cDNA 3′ ends (3′ RACE)

The 3′ extremities of NrsZ transcripts were determined by 3′-RACE according to the modified protocol of Argaman *et al*. (Argaman *et al*., 2001). Briefly, 40 μg of total RNA, extracted from the PAO1 WT strain grown in MMP supplemented with 20 mM glucose and 20 mM nitrate to an OD_{600} of ~ 2, was 5′-dephosphorylated with rAPID alkaline phosphatase (Roche Diagnostics). Dephosphorylated RNA was ligated

with 500 pmol of the 3′ RNA adapter P-RIBOLI (Table S1) using 50 U of T4 RNA ligase (New England Biolabs, Hertfordshire, England) in a buffer containing 50 mM HEPES/NaOH (pH 8.0), 20 mM $MgCl_2$, 3 mM DTT, 10 % (v/v) dimethyl sulfoxide (DMSO), 10 μg ml^{-1} BSA, 2 U RNasin (Promega) and 0.2 mM ATP in a final volume of 50 μl. Reverse transcription was carried out using Maxima Reverse Transcriptase (Fermentas, Pittsburgh, PA, USA) and the DEOXYLI primer complementary to the P-RIBOLI RNA adapter (Table S1) according to the manufacturer's recommendations but in the presence of 5% of DMSO. cDNAs complementary to NrsZ were PCR amplified (40 cycles with an annealing temperature of 57°C) using GoTaq polymerase (Promega) and primers DEOXYLI and NrsZ-3′RACE (Table S1). After agarose gel analyses of the resulting PCR products, the DNA fragments of interest were directly ligated into pGEM-T easy vector (Promega). Bacterial colonies obtained by transformation were screened for the presence of the expected fragments by PCR using the DEOXYLI and NrsZ-3′RACE primers, and plasmids carrying the inserts of interest were isolated and sequenced by the Sanger method using primer M13__40_long (Table S1).

RNA deep sequencing and qualitative analysis

Pseudomonas aeruginosa PAO1 was grown in MMP medium supplemented with 20 mM mannitol and 20 mM nitrate. Total RNA was isolated as previously mentioned. RNA size selection was performed following the modified protocol of Gómez-Lozano *et al*. (Gómez-Lozano *et al*., 2012). After separation on denaturing polyacrylamide gel (urea 10 M), two gel fractions corresponding to RNA molecules ranging from 30 nt to 200 nt and from 150 nt to 450 nt were excised, eluted in 0.4 M NaCl and treated with tobacco alkaline pyrophosphatase for 1 h at 37°C. Treated RNA samples were used to perform two multiplexed libraries according to the TruSeq Small RNA Sample Preparation kit (Illumina, San Diego, CA, USA) without fragmentation to preserve molecule integrity. High-GC Accuprime Polymerase (Invitrogen, New York, NY, USA) was used in PCR reactions to overcome elongation difficulties due to the high GC-content of the PAO1 transcriptome. 2×100 paired-end sequencing was performed on a TruSeq Illumina HiSeq2000 machine. After de-multiplexing, paired-end reads were mapped using bowtie (v0.2.19) (Langmead *et al*., 2009) and SAMTOOLS (v0.1.18) (Li *et al*., 2009) software on the PAO1 genome (NC_002516.2). 2×100 paired-end reads were merged to produce artificial orientated single-end reads ranging from 30 nt up to 450 nt.

β-Galactosidase assays

β-Galactosidase activities were determined by the Miller method (Miller, 1972) using cells grown at 37°C and permeabilized with 5% (v/v) toluene. All experiments were performed in triplicate.

Swarming motility assays

Swarming motility was assayed on MMP medium supplemented with 20 mM glucose and 0.1% (w/v) casamino acids plates solidified with 0.5% (w/v) bacteriological Agar (Oxoid) (modified from Yeung *et al*., 2011). Bacteria were grown in LB medium to mid-logarithmic-growth-phase (OD_{600} 0.5–1) and 1 μl of culture was plated in four independent replicates. Swarming was observed after 24 h of incubation at 37°C.

Rhamnolipids production assays

Rhamnolipids production was tested on the MMP medium supplemented with 20 mM glucose, 0.1% (w/v) casamino acids, 0.02% (w/v) cetyltrimethylammonium bromide and 0.0005% (w/v) methylene blue solidified with 1.2% (w/v) bacteriological agar (Oxoid) (modified from Pinzon and Ju, 2009). The plates were inoculated with single colonies grown overnight at 37°C on agar plates. Rhamnolipids production was estimated by measuring violet halos formed on plates around the colonies after 72 h incubation at 37°C, followed by an incubation at room temperature for the same duration.

Acknowledgements

We thank Cornelia Reimmann for providing the pME7549 plasmid, Sébastien Ritzman for helping in the construction of the plasmids pME10142-10145, and Christoph Keel, Onya Opota and Inés Canosa for providing the *P. protegens* Pf-5, *P. entomophila* L48 and *P. putida* KT2442 strains respectively. Karine Lapouge was supported by the Sandoz Family Foundation (Programme for Academic Promotion), the Swiss National Foundation for Scientific Research (Project 31003A-127587) and the Fondation Herbette.

References

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

Bao, Y., Lies, D.P., Fu, H., and Roberts, G.P. (1991) An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. *Gene* **109:** 167–168.

Barrios, H., Valderrama, B., and Morett, E. (1999) Compilation and analysis of σ^{54}-dependent promoter sequences. *Nucleic Acids Res* **27:** 4305–4313.

Blumer, C., Heeb, S., Pessi, G., and Haas, D. (1999) Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci USA* **96:** 14073–14078.

Breidenstein, E.B.M., de la Fuente-Núñez, C., and Hancock, R.E.W. (2011) *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* **19:** 419–426.

Buell, C.R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I.T., Gwinn, M.L., *et al*. (2003) The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc Natl Acad Sci USA* **100:** 10181–10186.

Caiazza, N.C., Shanks, R.M., and O'Toole, G.A. (2005) Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *J Bacteriol* **187:** 7351–7361.

Clarke, K.G., Ballot, F., and Reid, S.J. (2010) Enhanced rhamnolipid production by *Pseudomonas aeruginosa* under phosphate limitation. *World J Microbiol Biotechnol* **26:** 2179–2184.

Davis, B.M., and Waldor, M.K. (2007) RNase E-dependent processing stabilizes MicX, a *Vibrio cholerae* sRNA. *Mol Microbiol* **65:** 373–385.

Déziel, E., Lépine, F., Milot, S., and Villemur, R. (2003) *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. *Microbiology* **149:** 2005–2013.

Dixon, R., and Kahn, D. (2004) Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* **2:** 621–631.

Dötsch, A., Eckweiler, D., Schniederjans, M., Zimmermann, A., Jensen, V., Scharfe, M., *et al*. (2012) The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing. *PLoS ONE* **7:** e31092.

Ferrara, S., Brugnoli, M., De Bonis, A., Righetti, F., Delvillani, F., Dehò, G., *et al*. (2012) Comparative profiling of *Pseudomonas aeruginosa* strains reveals differential expression of novel unique and conserved small RNAs. *PLoS ONE* **7:** e36553.

Franklin, F.C.H., Bagdasarian, M., Bagdasarian, M.M., and Timmis, K.N. (1981) Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. *Proc Natl Acad Sci USA* **78:** 7458–7462.

Franze de Fernandez, M.T., Hayward, W.S., and August, J.T. (1972) Bacterial proteins required for replication of phage Qβ ribonucleic acid. Purification and properties of host factor I, a ribonucleic acid-binding protein. *J Biol Chem* **247:** 824–831.

Gómez-Lozano, M., Marvig, R.L., Molin, S., and Long, K.S. (2012) Genome-wide identification of novel small RNAs in *Pseudomonas aeruginosa*. *Environ Microbiol* **14:** 2006–2016.

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

Green, S.K., Schroth, M.N., Cho, J.J., Kominos, S.D., and Vitanzajack, V.B. (1974) Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **28:** 987–991.

Guerra-Santos, L., Käppeli, O., and Fiechter, A. (1984) *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. *Appl Environ Microbiol* **48:** 301–305.

Haas, D., Holloway, B.W., Schambock, A., and Leisinger, T. (1977) The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. *Mol Gen Genet* **154:** 7–22.

Hardalo, C., and Edberg, S.C. (1997) *Pseudomonas aeruginosa*: assessment of risk from drinking water. *Crit Rev Microbiol* **23:** 47–75.

Heckman, K.L., and Pease, L.R. (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc* **2:** 924–932.

Hervás, A.B., Canosa, I., and Santero, E. (2008) Transcriptome analysis of *Pseudomonas putida* in response to nitrogen availability. *J Bacteriol* **190:** 416–420.

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

Heurlier, K., Denervaud, V., Pessi, G., Reimmann, C., and Haas, D. (2003) Negative control of quorum sensing by RpoN (σ^{54}) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185:** 2227–2235.

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

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

Hutchison, M.L., and Govan, J.R.W. (1999) Pathogenicity of microbes associated with cystic fibrosis. *Microbes Infect* **1:** 1005–1014.

Kimata, N., Nishino, T., Suzuki, S., and Kogure, K. (2004) *Pseudomonas aeruginosa* isolated from marine environments in Tokyo Bay. *Microb Ecol* **47:** 41–47.

Köhler, T., Kocjancic Curty, L., Barja, F., van Delden, C., and Pechère, J.C. (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol* **182:** 5990–5996.

Kustu, S., Santero, E., Keener, J., Popham, D., and Weiss, D. (1989) Expression of σ^{54} (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiol Rev* **53:** 367–376.

Lang, S., and Wullbrandt, D. (1999) Rhamnose lipids – biosynthesis, microbial production and application potential. *Appl Microbiol Biotechnol* **51:** 22–32.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10:** R25.

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

Lease, R.A., and Woodson, S.A. (2004) Cycling of the Sm-like protein Hfq on the DsrA small regulatory RNA. *J Mol Biol* **344:** 1211–1223.

Leoni, L., Ciervo, A., Orsi, N., and Visca, P. (1996) Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of fur and PvdS on promoter activity. *J Bacteriol* **178:** 2299–2313.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., *et al*. (2009) The sequence alignment/map format and SAMtools. *Bioinformatics* **25:** 2078–2079.

Li, W., and 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.

Maier, R.M., and Soberón-Chávez, G. (2000) *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Appl Microbiol Biotechnol* **54:** 625–633.

Medina, G., Juárez, K., Diaz, R., and Soberón-Chávez, G. (2003a) Transcriptional regulation of *Pseudomonas aeruginosa* rhlR, encoding a quorum-sensing regulatory protein. *Microbiology* **149:** 3073–3081.

Medina, G., Juárez, K., Valderrama, B., and Soberón-Chávez, G. (2003b) Mechanism of *Pseudomonas aeruginosa* RhlR transcriptional regulation of the *rhlAB* promoter. *J Bacteriol* **185:** 5976–5983.

Merrick, M.J., and Edwards, R.A. (1995) Nitrogen control in bacteria. *Microbiol Mol Biol Rev* **59:** 604–622.

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

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

Mulligan, C.N., and Gibbs, B.F. (1989) Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **55:** 3016–3019.

Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Dos Santos, V.A.P., *et al*. (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4:** 799–808.

Ninfa, A.J., Atkinson, M.R., Kamberov, E.S., Feng, J.L., and Ninfa, E.G. (1995) Control of nitrogen assimilation by the NRI-NRII two-component system of enteric bacteria. In *Two-Component Signal Transduction*. Hoch, J.A., and Silhavy, T.J. (eds). Washington, DC, USA: American Society for Microbiology Press, pp. 67–88.

Nishijyo, T., Haas, D., and 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.

Ochsner, U.A., Fiechter, A., and Reiser, J. (1994) Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *J Biol Chem* **269:** 19787–19795.

Overhage, J., Lewenza, S., Marr, A.K., and Hancock, R.E.W. (2007) Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn*5-lux* mutant library. *J Bacteriol* **189:** 2164–2169.

Overhage, J., Bains, M., Brazas, M.D., and Hancock, R.E.W. (2008) Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol* **190:** 2671–2679.

Papenfort, K., Said, N., Welsink, T., Lucchini, S., Hinton, J.C., and Vogel, J. (2009) Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA. *Mol Microbiol* **74:** 139–158.

Paulsen, I.T., Press, C.M., Ravel, J., Kobayashi, D.Y., Myers, G.S.A., Mavrodi, D.V., *et al*. (2005) Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat Biotechnol* **23:** 873–878.

Pearson, J.P., Pesci, E.C., and Iglewski, B.H. (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179:** 5756–5767.

Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H. (1997) Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179:** 3127–3132.

Pinzon, N.M., and Ju, L.K. (2009) Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. *Biotechnol Lett* **31:** 1583–1588.

Potvin, E., Sanschagrin, F., and Levesque, R.C. (2008) Sigma factors in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* **32:** 38–55.

Rashedi, H., Jamshidi, F., Mazaheri Assadi, M., and Bonakdarpour, B. (2005) Isolation and production of biosurfactant from *Pseudomonas aeruginosa* isolated from Iranian southern wells oil. *Int J Environ Sci Technol* **2:** 121–127.

Rice, P., Longden, I., and Bleasby, A. (2000) EMBOSS: the European molecular biology open software suite. *Trends Genet* **16:** 276–277.

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

Romeo, A., Sonnleitner, E., Sorger-Domenigg, T., Nakano, M., Eisenhaber, B., and Bläsi, U. (2012) Transcriptional regulation of nitrate assimilation in *Pseudomonas aeruginosa* occurs via transcriptional antitermination within the *nirBD-PA1779-cobA* operon. *Microbiology* **158:** 1543–1552.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (2001) *Molecular Cloning: A Laboratory Manual (Third Edition)*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., *et al*. (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.

Sharma, C.M., and Vogel, J. (2009) Experimental approaches for the discovery and characterization of regulatory small RNA. *Curr Opin Microbiol* **12:** 536–546.

Soberón-Chávez, G., Lépine, F., and Déziel, E. (2005) Production of rhamnolipids by *Pseudomonas aeruginosa*. *Appl Microbiol Biotechnol* **68:** 718–725.

Sonnleitner, E., and Haas, D. (2011) Small RNAs as regulators of primary and secondary metabolism in *Pseudomonas* species. *Appl Microbiol Biotechnol* **91:** 63–79.

Sonnleitner, E., Abdou, L., and 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., González, N., and Haas, D. (2010) Small RNAs of *Pseudomonas* spp. In *Pseudomonas—Molecular Microbiology, Infection and Biodiversity. Vol. 6*. Ramos, J.-L., and Filloux, A. (eds). Dordrecht, The Netherlands: Springer, pp. 3–28.

Sonnleitner, E., Romeo, A., and Bläsi, U. (2012) Small regulatory RNAs in *Pseudomonas aeruginosa*. *RNA Biol* **9:** 364–371.

Sonnleitner, E., Schuster, M., Sorger-Domenigg, T., Greenberg, E.P., and Blasi, U. (2006) Hfq-dependent

alterations of the transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*. *Mol Microbiol* **59:** 1542–1558.

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

Sonnleitner, E., Sorger-Domenigg, T., Madej, M.J., Findeiss, S., Hackermuller, J., Huttenhofer, A., *et al*. (2008) Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. *Microbiology* **154:** 3175–3187.

Storz, G., Vogel, J., and Wassarman, K.M. (2011) Regulation by small RNAs in bacteria: expanding frontiers. *Mol Cell* **43:** 880–891.

Vodovar, N., Vallenet, D., Cruveiller, S., Rouy, Z., Barbe, V., Acosta, C., *et al*. (2006) Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. *Nat Biotechnol* **24:** 673–679.

Voisard, C., Bull, C., Keel, C., Laville, J., Maurhofer, M., Schnider, M., *et al*. (1994) Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In *Molecular Ecology of Rhizosphere Microorganisms*. O'Gara, F., Dowling, D.N., and Boesten, B. (eds). Weinheim, Germany: VCH Publishers, pp. 67–89.

Waters, L.S., and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell* **136:** 615–628.

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

Yeung, A.T., Bains, M., and Hancock, R.E. (2011) The sensor kinase CbrA is a global regulator that modulates metabolism, virulence, and antibiotic resistance in *Pseudomonas aeruginosa*. *J Bacteriol* **193:** 918–931.

Yeung, A.T., Torfs, E.C., Jamshidi, F., Bains, M., Wiegand, I., Hancock, R.E., and Overhage, J. (2009) Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. *J Bacteriol* **191:** 5592–5602.

Zhang, X.X., and Rainey, P.B. (2008) Dual involvement of CbrAB and NtrBC in the regulation of histidine utilization in *Pseudomonas fluorescens* SBW25. *Genetics* **178:** 185–195.

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

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Schematic of the *nrsZ* locus. The sequence of *nrsZ* from its putative +1 transcription start site to the *PA5126* gene stop codon is indicated in bold. The RpoN promoter of *nrsZ* is boxed, and the invariant –24 and –12 nucleotides are underlined. The RpoN promoter sequence degenerated in the strain PAO6846 (*nrsZ*$^-$) is indicated in a grey box. The promoter region cloned in the *nrsZ-lacZ* is indicated and encompasses the sequence from the T nucleotide labelled by a star to the vertical arrow.

Fig. S2. *nrsZ-lacZ* expression in PAO1 WT strain grown with different casamino acids concentration. The PAO1 WT strain carrying *nrsZ-lacZ* (PAO6750) was inoculated at an initial optical density (OD_{600}) of 0.05, and was grown in MMP supplemented with glucose as carbon source and casamino acids (CA) at different concentrations [0.05%, 0.1%, 0.2% or 0.5% (w/v)]. The growth (OD_{600}) (A) and the activity of the *nrsZ-lacZ* chromosomal fusion (B) were followed in the different conditions during 10.5 h of incubation. Each value represents the average of triplicate cultures ± standard deviation.

Fig. S3. NrsZ promoter and structure are conserved among Pseudomonads.

A. Alignment of the promoters of *nrsZ* in different Pseudomonads. The RpoN promoter sequence identified in *P. aeruginosa* PAO1 is in bold. Nucleotides at –12 and –24 are in red box, and conserved nucleotides are indicated with a star. The predicted transcriptional start site of NrsZ in PAO1 and other Pseudomonads is in bold. The matured 5′ extremity of the first stem loop is indicated by an arrow.

B. Predicted secondary structures of NrsZ in different Pseudomonads (using the MFOLD software). The conserved motifs of NrsZ are in red, and the unconserved spacer sequences represented by a square.

Fig. S4. Rhamnolipids production in the *nrsZ*$^-$ mutant strain is restored by *nrsZ* of other Pseudomonads. Rhamnolipids production was assayed as described in the *Experimental procedures* for strains PAO1 WT and PAO1 *nrsZ*$^-$ (PAO6846) containing the empty vector pME4510, or its derivative plasmids pME9995 (p$nrsZ_{PAO1}$), pME10142 (p$nrsZ_{Pf\text{-}5}$), pME10143 (p$nrsZ_{KT2442}$), pME1044 (p$nrsZ_{L48}$) and pME10145 (p$nrsZ_{DC3000}$), carrying respectively the *nrsZ* genes of *P. aeruginosa* PAO1, *P. protegens* Pf-5, *P. putida* KT2442, *P. entomophila* L48 and of *P. syringae* pv. tomato DC3000, under the control of their native promoter.

Table S1. List of primers used in this study.