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

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

INTRODUCTION

During the last few years an increasing number of non-coding RNAs (ncRNAs) and small regulatory RNAs (sRNAs) has been described in different pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae* and *Pseudomonas aeruginosa* (strain PAO1) (Argaman et al., 2001; Sittka et al., 2007; Heurlier et al., 2004; Lenz et al., 2004; Wilderman et al., 2004; Zhang et al., 2003). Some of these sRNAs are involved in quorum sensing (QS) and bacterial pathogenesis, such as RsmY and RsmZ in PAO1 (Heurlier et al., 2004; Sonnleitner et al., 2006) and Qtr1-4 in V. *cholerae* (Lenz et al., 2004). Another group of sRNAs modulates the stress response by regulating the expression of the alternative sigma factor σ^54 (Repoila et al., 2003), while others are important for iron metabolism (Massé & Gottesman, 2002; Wilderman et al., 2004), catabolite regulation (Møller et al., 2002) and for remodelling of the outer membrane (Guillier et al., 2006). A large number of sRNAs has been shown to associate with Hfq and to require this protein for post-transcriptional regulation (Gottesman, 2005; Massé et al., 2003). Part of this requirement can be explained by Hfq-mediated stabilization of some of the studied sRNAs, e.g. DsrA, Spot42 RNA, RyhB and RsmY (Møller et al., 2002; Moll et al., 2003a; Sledjeski et al., 2001; Sonnleitner et al., 2006), as well as by the RNA chaperone function of Hfq (Moll et al., 2003b), which facilitates the interaction of sRNAs with their target mRNAs (Kawamoto et al., 2006; Zhang et al., 2002).

Hfq has been identified as a virulence factor in *Yersinia enterocolitica*, *Brucella abortus*, *Legionella pneumophila*, *Salmonella typhimurium* and *V. cholerae* (Sittka et al., 2007; Ding et al., 2004; McNealy et al., 2005; Nakao et al., 2006).
1995; Robertson & Roop, 1999). A P. aeruginosa PAO1 hfq—mutant showed a reduced virulence in larvae of Galleria mellonella and in mice (Sonnleitner et al., 2003). As in E. coli (Sledjeski et al., 2001), Hfq is involved in regulation of rpoS expression in PAO1 (Sonnleitner et al., 2003). However, Hfq also exerts s^2-independent effects on catalase, pyocyanin and elastase production, and is required for PAO1 to swarm and twitch, which are important traits for colonization and biofilm development (Sonnleitner et al., 2003). A comparative transcriptome analysis of a PAO1 rpoS− and a rpoS−hfq− strain has indicated that Hfq affects approximately 5% of the PAO1 transcripts (Sonnleitner et al., 2006). Among these transcripts, 72 are known to be regulated by QS, which is a cell density-dependent regulatory mechanism, impacting on virulence gene expression (Van Delden & Iglewski, 1998). Hfq has been suggested to affect QS and QS-controlled genes in at least two ways: by a non-specific positive regulation of the QS repressor QscR and of the pqshg gene, and by RsmY-mediated indirect positive regulation of the QS regulator Rhf (Sonnleitner et al., 2006).

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

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

**METHODS**

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

**RNomics.** Total RNA was extracted from PAO1 cultures grown to early stationary phase (LB medium; OD₆₀₀ = 2) and from PAO1 cultures, which were grown in 200 ml LB to OD₆₀₀ 1.0, followed by further incubation for 4 h in 50 ml PBS buffer (160 mM NaCl, 4 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) containing 15% non-inactivated human serum HS-AB-100 (PromoCell). The cells were resuspended in VD buffer (10 mM Tris/HCl, pH 7.4, 6 mM NH₄Cl, 6 mM β-mercaptoethanol) containing 2 mM magnesium acetate, and lysed by sonication. The extracts were centrifuged at 150,000 × g for 6 h to pellet ribosomes and rRNA. The supernatant was treated with DNase I (Fermentas), and the RNA was purified by phenol/chloroform extraction. Total RNA was size-fractionated on denaturing 6% polyacrylamide/8 M urea gels. RNAs in the range

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype/relevant features</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type</td>
<td>Holloway et al. (1979)</td>
</tr>
<tr>
<td>PAO1 hfq−</td>
<td>PAO1 hfq::aadA, Sm/Sp^R</td>
<td>Sonnleitner et al. (2003)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>Top10</td>
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<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pGEM-T Vector</td>
<td>Broad–host-range promoter-probe plasmid, Gm^R</td>
<td>Promega</td>
</tr>
<tr>
<td>pME4510</td>
<td>pME4510 harbouring phrD (authentic promoter)</td>
<td>Rist &amp; Kertesz (1998)</td>
</tr>
<tr>
<td>pMEphrD</td>
<td>pME4510 harbouring phrS (authentic promoter)</td>
<td>This study</td>
</tr>
<tr>
<td>pMEphrS</td>
<td>colE1 ori, Amp^R</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19</td>
<td>pUC19 bearing phrD under T7 promoter control</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC19-TphrD</td>
<td>pUC19 bearing phrS under T7 promoter control</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19-TphrS</td>
<td>Cloning vectors for translational lacZ fusions, Tr^R</td>
<td>This study</td>
</tr>
<tr>
<td>pME6013/14/15</td>
<td>pME6013 with phrS-ORF^−−lacZ</td>
<td>Schneider-Kee et al. (2000)</td>
</tr>
<tr>
<td>pME9651</td>
<td>pME6013 with phrS-ORF^AVG−4CUG−−lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pME9651-1</td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>
between ~50 and 500 nt were excised from the gel, eluted and ethanol-purified. Ten micrograms of gel-purified RNA was added to 1 nmol purified PAO1 Hfq. After 10 min on ice, 10 μl polyclonal rabbit anti-Hfq IgG was added and incubation was extended for 45 min on ice. Then, 20 μl Dynabeads Protein G (Dynal Biotech), washed once with 0.1 M sodium acetate and twice with VD buffer containing 2 mM magnesium acetate, was added. After 40 min at room temperature the mixture was subjected to a magnetic device (Dynal MPC) to capture Hfq-bound RNAs. To remove unbound components, the beads were washed four times with VD buffer containing 2 mM magnesium acetate and 480 mM NH₄Cl. The Hfq-bound RNAs were eluted from the beads by phenol/chloroform extraction, and subsequently precipitated with ethanol. For the cDNA library, the RNAs were poly-C-tailed by poly(A) polymerase (Invitrogen), the C-tailed RNAs were ligated to a 5’ oligonucleotide linker, and the RNAs were then converted into cDNA by RT-PCR, as described by Hüttenhofer & Vogel (2006), employing primers complementary to the 5’ linker and the poly(C) tail, followed by cloning into the pGEM-T vector (Promega). The cDNA clones were sequenced (Lung et al., 2006), and the cDNA sequences were compared with one another using the Lasergene Seqman II program package to identify identical sequences (DNASTAR). Following a BLASTN search against the PAO1 genome (http://www.pseudomonas.com), all sequences corresponding to intergenic regions or to the opposite strand of protein-coding regions were considered to encode potential novel sRNAs/ncRNAs.

Construction of plasmids and RNA preparation. For in vitro transcription of PhrD and PhrS, the plasmids pUC19-T7phrD and pUC19-T7phrS (Table 1) were constructed by inserting the PCR products generated with primer pair Z32 and J33 (phrD Supplementary Table S1), and primer pair J33 and I33 (phrS; Supplementary Table S1), respectively, into the XbaI–PstI site of plasmid pUC19. The forward primers Z32 and J33 contained a T7 promoter sequence.

The plasmids pME9651 and pME9651-1, harbouring the phrS-ORF2′–lacz and the phrS-ORF1 Ala1–Asp9′–lacz fusion genes, respectively, were constructed as follows. The promoter region of phrS and the first 47 bp of phrS were amplified (Fig. 1) using primer C2, phrSlacZfw together with primer D2, phrSlacZrev or primer O3, phrStop (Supplementary Table S1) and genomic DNA of PAO1 as template. The PCR fragments were digested with EcoRI and PstI and ligated into the corresponding sites of pME6013 (Table 1), resulting in pME9651 and pME9651-1, respectively.

For overproduction of the PhrD and PhrS RNAs, the plasmids pMEphrD and pMEphrS (Table 1) were constructed. The respective DNA sequences of phrD and phrS, including their authentic promoters, were amplified by PCR using the primer pairs T33 and Y32 for phrD and H33 and J33 for phrS (Supplementary Table S1), containing a BamHI or PstI restriction site (highlighted in bold type in Supplementary Table S1). The PCR fragments were then cloned into the corresponding sites of plasmid pME4510 (Table 1), resulting in pMEphrD and pMEphrS, respectively.

Northern blotting. Total RNA of PAO1 and PA01inhf– was purified using the hot phenol method (Lin-Chao & Bremer, 1986). The abundance of the PhrD and PhrS RNAs was determined by Northern blotting using 15 μg total RNA. The signals were normalized to the hybridization signal obtained for ribosomal 5S rRNA, as described by Sonnleitner et al. (2006). The RNA samples were denatured for 5 min at 65 °C in loading buffer containing 50% formamide, separated on an 8% polyacrylamide/8 M urea gel and then transferred to nylon membranes by electroblotting. The RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized with gene-specific 32P-end-labelled oligonucleotides (PhrD, P31; PhrS, O30; 5S rRNA, J26; see Supplementary Table S1), and the hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

Determination of the half-lives of PhrD and PhrS in PAO1 and PAO1inhf–. PAO1 and PAO1inhf– were grown in LB medium to OD₆₀₀ 2.0, then rifampicin (final concentration 100 μg ml⁻¹) was added to both strains and 10 ml aliquots were withdrawn at 0, 15, 30 and 45 min thereafter for isolation of total RNA (see above). Total RNA (6 μg) of each sample was loaded on an 8% polyacrylamide/8 M urea gel and blotted to a Hybond-N membrane (Amersham). PhrD and PhrS were visualized with DIG-labelled double-stranded probes. Chromosomal RNA of PAO1 was used as a template for the DIG-labelling procedure, as described by the manufacturer (DIG DNA Labeling Mix, Roche) with the primer pairs PhrDfw and PhrDrev for PhrD, PhrSfw and PhrSrev for PhrS, and 5S-rRNA-1 and 5S-rRNA-2 for 5S rRNA (Supplementary Table S1), which served as a loading control.

Primer extension analysis. For determination of the 5’ ends of PhrD and PhrS (Fig. 2), and PhrX and PhrY (Supplementary Fig. S1), total RNA was purified using the hot phenol method (Lin-Chao & Bremer, 1986) after growth of PAO1 in LB medium to OD₆₀₀ 2.0. Primer extension was performed with AMV reverse transcriptase (Promega) using 2 μg total RNA and the 5’ end-labelled oligonucleotides P31 for PhrD, O30 for PhrS, I37 and B32 for PhrX, and K37 and R30 for PhrY (Supplementary Table S1). The plasmids pMEphrD and pMEphrS were used as templates in DNA sequencing reactions. To verify overexpression of the plasmid-encoded PhrS RNA, total RNA was purified from PAO1 harbouring plasmids pME4510 or pMEphrS in LB medium during exponential growth (OD₆₀₀ = 0.8), or upon growth to early stationary (OD₆₀₀ = 1.5) or stationary phase (OD₆₀₀ = 2.5). The primer extension experiments were carried out as mentioned above, and the normalization of the primer extension signal for PhrS to the 5S rRNA signal was performed as described by Sonnleitner et al. (2006).

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

Gel mobility shift assays. The PhrD and PhrS RNAs were transcribed in vitro using T7 polymerase (Fermentas) and the Pri-linearized plasmids pUC-T7phrD and pUC-T7phrS, as described by Sonnleitner et al. (2006). The in vitro-transcribed RNAs were 5’ end-labelled with [γ-32P]ATP (Amersham) and purified on 6% polyacrylamide/8 M urea gels. Labelled RNA (0.05 pmol) was incubated with increasing amounts of purified Hfq hexamer protein (Hfq6), as described by Sonnleitner et al. (2006). Non-labelled PhrD, PhrS and RsmY RNA, respectively, were used as specific competitors, and E. coli bulk RNA as a non-specific competitor. Immediately before loading,
the samples were mixed with 4 μl loading dye (25% glycerol, 0.2 mg xylene cyanol 1−1 and bromphenol blue), and loaded on a native 4% polyacrylamide gel. Electrophoresis was performed in Tris-acetate/EDTA buffer at 160 V. The radioactively labelled bands were visualized with a PhosphorImager (Molecular Dynamics).

2D gel analysis. To detect PhrS-mediated regulatory effects, differences in the proteome profile were analysed, upon plasmid-directed overexpression of PhrS RNA. Total cellular extracts of strain PAO1 harbouring plasmid pME4510 or pMEphrS were extracted and compared by 2D gel electrophoresis. The experiment was performed twice to ensure reproducibility. The strains were grown in LB medium to OD600 1.5, and equal amounts of cells were dissolved in lysis buffer [8 M urea, 4% (w/v) CHAPS, 40 mM Tris base]. The cells were disrupted by repeated freezing in liquid N2 and thawing at 37 °C, followed by incubation at 37 °C for 1 h to achieve complete lysis. For the first dimension, the Immobiline Dry Strip pH 3–10 (18 cm; Amersham Pharmacia Biotech) was used with the following IEF program: 12 h rehydration at 40 V, 0.5 h at 300 V, 0.5 h at 1000 V, 0.5 h at 2000 V, 0.5 h at 3000 V, 9 h at 7000 V at 18 °C (IPGphor isoelectric focusing system). Resolution in the second dimension was performed on 12% SDS-polyacrylamide gels for 15 min at 15 mA, and then for 4 h at 20 mA. Buffers and conditions were used according to the manufacturer’s instructions. The gels were silver-stained as described elsewhere (Shevchenko et al., 1996). Selected protein spots were excised from the gel and the protein identities were assessed by MS.

RNAz screen. The tool NcDNAlign (Rose et al., 2008) was used to construct multiple sequence alignments based on the known genome sequences of P. aeruginosa PAO1 (NC 002516), P. aeruginosa UCBPP-PA14 (NC 008463), Pseudomonas entomophila (NC 008027), Pseudomonas fluorescens Pf-5 (NC 004129), P. fluorescens PfO-1 (NC 007492), Pseudomonas putida KT2440 (NC 002947), Pseudomonas syringae pv. phaseolicola 1448A (NC 005773), P. syringae (NC 007005), P. syringae pv. tomato str. DC3000 (NC 004578) and P. fluorescens SBW25 (ftp://ftp.sanger.ac.uk/pub/pathogens/pf/PF.dbs). Briefly, the program is based on pairwise BLASTN (Altschul et al., 1990).
(e-value $<10^{-3}$) comparisons, which are combined to multiple alignments. For comparison, MultiZ (Blanchette et al., 2004) was additionally used to produce an alternative set of genome-wide alignments. Both alignments were used as input for the RNAz pipeline (Washietl et al., 2005a, b). The RNAz approach searches for signatures of conservation of RNA secondary structure in a multiple sequence alignment and uses a support vector machine (SVM) to distinguish conserved RNA structure elements from genomic background. In order to assess the reliability of the predictions, the procedure was repeated two times with two different sets of input alignments. In each case the procedure outlined in the current version of the RNAz manual was used (Washietl, 2006). As the SVM of RNAz cannot handle more than six aligned sequences, from alignments exceeding six, the pipeline selects a subset of six sequences that have approximately equal pairwise sequence similarities. Input alignments are cut into windows of length 120 with 40 nt overlap between adjacent slices, which are scored individually. Finally, the predictions are combined to contiguous structured loci at the reference genome, in this case PAO1. As RNAz returns a classification confidence, we report the results for two cutoff values: $p > 0.5$ and a high confidence set with $p > 0.9$. The predicted structured RNAs were compared using BLASTN to the available public databases, the sequences reported by Livny et al. (2006) and Gonzalez et al. (2008), and the Hfq-binding RNAs listed in Table 2. To identify known PAO1 ncRNAs among the predictions, mazAnnotate.pl and a minimum overlap of 0.7 were used.

**RESULTS AND DISCUSSION**

**Identification of ncRNAs and sRNA candidates by Rnomics**

With the aim of identifying sRNAs in PAO1, we used an Rnomics approach, which is based on the generation of specialized cDNA libraries encoding potential novel sRNA species (Hüttenhofer et al., 2004; Hüttenhofer & Vogel, 2006; Vogel et al., 2003). In an initial attempt, we shotgun-cloned size-fractionated total RNA (50–300 nt). However, as revealed by subsequent sequencing, this approach yielded mainly RNA fragments originating from rRNA and tRNA (not shown). In a second experiment, a cell extract was used for co-immunoprecipitation of Hfq-binding RNAs with PAO1-Hfq specific antibodies upon...
Table 2. RNAs mapping to intergenic regions of PAO1

<table>
<thead>
<tr>
<th>sRNA*</th>
<th>Adjacent genes</th>
<th>Strand†</th>
<th>Growth‡</th>
<th>5’ end§</th>
<th>3’ end</th>
<th></th>
<th>Length¶</th>
<th>Identified sequence#</th>
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</thead>
<tbody>
<tr>
<td>RsmY</td>
<td>dnr/PA0528</td>
<td>← → → ←</td>
<td>S</td>
<td>586 867</td>
<td>586 990</td>
<td>124</td>
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<tr>
<td>PhrC</td>
<td>PA0667/tyrZ</td>
<td>← → → ←</td>
<td>LB</td>
<td>720 082</td>
<td>720 136</td>
<td>72</td>
<td>720 082–720 136</td>
<td></td>
</tr>
<tr>
<td>PhrR</td>
<td>PA0714/PA0715</td>
<td>→ → → →</td>
<td>S/LB</td>
<td>785 497</td>
<td>785 570</td>
<td>72</td>
<td>785 498–785 547</td>
<td></td>
</tr>
<tr>
<td>tmRNA</td>
<td>PA0826/PA0827</td>
<td>← ← ← ←</td>
<td>LB</td>
<td>901 536</td>
<td>901 640</td>
<td>72</td>
<td>901 536–901 640</td>
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<td>PhrR</td>
<td>mobAPA3031</td>
<td>← → → ←</td>
<td>LB</td>
<td>3 394 727–3 394 805</td>
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<tr>
<td>PhrS(P20/1887)**</td>
<td>PA3305/PA3306</td>
<td>← ← ← ←</td>
<td>LB</td>
<td>3 705 522–3 705 309</td>
<td>212</td>
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<tr>
<td>amiE leader</td>
<td>amiE/PA3367</td>
<td>← ← ← ←</td>
<td>LB</td>
<td>3 778 134–3 778 034</td>
<td>100</td>
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<td>PhrU</td>
<td>PA3868/PA3869</td>
<td>← ← ← ←</td>
<td>S</td>
<td>4 332 627–4 332 676</td>
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<tr>
<td>PhrW(P2/2510)**</td>
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<td>← ← ← ←</td>
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<td>4 956 348–4 956 591</td>
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<tr>
<td>PhrXRT-PCR</td>
<td>PA5183/PA5184</td>
<td>← ← ← ←</td>
<td>S</td>
<td>5 836 429–5 836 579</td>
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<td>5 859 480–5 859 674</td>
<td>195</td>
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</tbody>
</table>

*New sRNA candidates revealed by RNomics detected by Northern-blotting (NB) or by RT-PCR (RT-PCR) are highlighted in bold type.
†The middle arrow indicates the orientation of the RNAs, while the flanking arrows indicate the orientation of the adjacent genes. Genes present on the strand given in the PAO1 genome database (http://v2.pseudomonas.com) (←); genes present on the complementary strand (→).
§RNA fragments were detected in PAO1 grown to early stationary phase in LB medium (LB) and/or after serum exposure (S) as described in Methods.
¶Determined by primer extension.
#Identified by RNomics, the numbers represent the location on the PAO1 genome (http://v2.pseudomonas.com).

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

A total of 400 cDNA sequences were analysed by first grouping identical cDNA clones, followed by their location by bioinformatics on the PAO1 genome. Although the cell extracts were centrifuged at 150 000 g to remove ribosomes, ~15% of the sequenced clones from stationary-phase or serum-treated cells represented rRNA fragments. Of the clones, 40 and 20% were derived from coding regions of PAO1 (presumably representing mRNA degradation intermediates) and from the opposite strand of protein-coding regions, respectively. Eleven candidate sRNA-encoding genes (Table 2) were predicted to localize to intergenic regions based on the presence of putative rho-independent terminators.
serum. Only two *Pseudomonas* Hfq-binding RNAs, termed PhrD and PhrS (Fig. 1a, b), isolated from cells exposed to serum and from stationary-phase cells, respectively, were detected (Fig. 2a, b, Table 2). PhrS matched with the recently described, but not further characterized, sRNA P20/1887 (Livny et al., 2006; González et al., 2008). From the determination of their major 5’ ends by primer extension (Fig. 2c, d), and from the position of the putative transcriptional terminator, the sizes of these sRNAs were estimated to be 72 nt (PhrD) and 212 nt (PhrS), which was in agreement with the size of the corresponding signals detected on Northern blots (Fig. 2a, b). A putative −10 box (TATGAT) and a putative −35 box (TTGTGC) were found upstream of the predicted transcription start site of phrD. A putative σ70 promoter with a −10 box (TAATCT) and a −35 box (TTGTGC) was likewise detected upstream of the transcriptional start site of phrS (Fig. 2e). However, with only 13 bp, the spacing between the −35 and the −10 region would deviate from that of canonical σ70 promoters. Nonetheless, as shown below (Fig. 5), this putative promoter was able to direct transcription of the plasmid-borne phrS gene. A BLASTN search of PhrD with all bacterial sequences in the NCBI homepage (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) revealed that PhrD is only present in PAO1, whereas homologues of PhrS are found in different isolates of *P. aeruginosa* (PACS2, C3719, 2192, UCBPP-PA14 and PA7), although not in other Gram-negative bacteria. Among the remaining five candidate sRNA genes, the transcription of phrX and phrY sequences could be verified by RT-PCR (Supplementary Figs S1 and S2a, b). PhrX and PhrY were isolated from cDNA libraries generated from cells exposed to human serum and from cells grown to early stationary phase, respectively. From the primer extension analyses (Supplementary Fig. S2c), and from the position of the putative transcriptional terminator, the sizes of these RNAs were calculated to be 151 nt (PhrX; Supplementary Fig. S1) and 174 nt (PhrY; Supplementary Fig. S1), respectively. PhrX and PhrY are transcribed from the intergenic regions between PA5183 and PA5184 (Supplementary Fig. S1a, Table 2) and between argA, encoding an *N*-acetylglutamate synthase, and PA5205, encoding a conserved hypothetical membrane protein (Supplementary Fig. S1b, Table 2), respectively.

**PhrS contains an ORF**

Inspection of the *phrS* gene revealed an ORF with the capacity to encode a 37 aa peptide (Fig. 1b). To date, only a few regulatory RNAs have been shown to have protein-coding capacity. For instance, the RNAIII gene of *Staph. aureus* contains an ORF *hld*, which encodes delta-haemolsyn (Janzon & Arvidson, 1990), and the *E. coli* SrgS sRNA, besides being a riboregulator, encodes the 43 aa SgrT polypeptide with a function in glucose uptake (Wadler & Vanderpool, 2007). To test whether the internal ORF of *phrS* (*phrS*-ORF) is expressed, a translational fusion between the *phrS*-ORF and the *lacZ* reporter gene was engineered. In plasmid pME9651 transcription of the *phrS*-ORF–*lacZ* gene is driven by the authentic *phrS* promoter. The control plasmid pME9651-1, which harbours the same *phrS*-ORF–*lacZ* gene, but wherein the start codon of *phrS*-ORF is changed to a CUG (*phrS*-ORF<sub>AUG→CUG→lacZ</sub>), did not direct synthesis of the fusion protein. In contrast, plasmid pME9651 directed synthesis of the PhrS<sub>F0</sub>LacZ protein (Fig. 1b, inset), indicating that the internal ORF of *phrS* is indeed translated. Thus, PhrS appears to be another candidate for a bifunctional sRNA acting as a riboregulator (see below) and as mRNA. The function of the encoded peptide remains to be elucidated.

**Binding of PhrD and PhrS to Hfq**

As PhrD and PhrS were abundant RNA species (Fig. 2), we focused in further experiments on these sRNAs. First, we verified that they bind to Hfq by performing band-shift assays with purified PAO1 Hfq protein. Hfq<sub>6</sub> was added in increasing molar ratios to 5 nM of the respective 5’ end-labelled RNA. When Hfq<sub>6</sub> was added to PhrD in a molar ratio of 1:1, two band shifts were observed (Fig. 3a, lane 2; B1 and B2). However, the amount of PhrD present in shift B2 was only marginal and did not increase with higher concentrations of Hfq<sub>6</sub>. The apparent *Kd* value was 5.5 ± 3.7 nM when 50% of PhrD RNA was present in complex B1. The competition experiment suggested that PhrD binds specifically to Hfq. Unlabelled PhrD RNA competed with the Hfq–PhrD complex (Fig. 3a, lane7) when added in twofold molar excess over Hfq, whereas the non-specific competitor *E. coli* tRNA did not (Fig. 3a, lanes 8–9).

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

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

To test whether Hfq affects the abundance of PhrD and PhrS, their steady-state levels were assessed by Northern blotting using total RNA isolated from PAO1, from PAO1<sub>hfq<sup>−</sup></sub> grown in LB medium to OD<sub>600</sub> 2 (Fig. 4), or after growth in LB to OD<sub>600</sub> 1.0, followed by exposure to non-inactivated human serum in PBS buffer (Serum; Fig. 4). When compared with PAO1, the steady-state level of PhrD was reduced ~50% in the *hfq<sup>−</sup>* strain under both conditions.
conditions (Fig. 4a). PhrS was predominantly synthesized in early stationary phase, and was absent upon serum exposure (Fig. 4b). When compared to PAO1, the steady-state level of PhrS was ~50% reduced in the PAO1 hfq \(^2\) mutant (Fig. 4b). These experiments indicated that Hfq affects the abundance of PhrD and PhrS, which could result from direct or indirect effects of Hfq on transcription of the two sRNAs, or from Hfq-mediated protection of these sRNAs from degradation (Moll et al., 2003a; Sorger-Domenigg et al., 2007). To distinguish between these possibilities, we next determined the half-lives of both sRNAs. The half-lives of PhrD and PhrS were comparable in PAO1 and in the PAO1 hfq \(^2\) mutant strain (Fig. 4c). Hence, Hfq appears to affect expression of these sRNAs rather than stabilizing them.

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

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

**Proteome analyses upon overproduction of PhrS**

Under the premise that riboregulation by an sRNA can result in a decreased or increased synthesis of the protein encoded by the target mRNA, plasmid-directed overexpression of phrS followed by proteomics was used as a means to identify putative PhrS targets. When compared to chromosome-directed background synthesis in strain
PAO1(pME4510), the levels of PhrS were 12-fold increased in strain PAO1(pMEphrS) when the cells entered early stationary phase (Fig. 5a, lanes 3 and 4). Therefore, samples for 2D gel analysis were withdrawn at OD600 1.5. The 2D protein pattern of PAO1(pME4510) was compared to that of PAO1(pMEphrS) (Fig. 5b), and three distinct protein spots were selected for identification by MS. The three possible targets of PhrS included the heat-shock chaperonin GroEL, the outer membrane porin OprD and the putative periplasmic binding protein PA5153, all of which were upregulated in the presence of increased levels of PhrS (Fig. 5b). GroEL is among the most highly conserved proteins in nature (Segal & Ron, 1996), and functions together with GroES to maintain protein integrity, which enables cells to survive a variety of environmental stresses (Hendrick & Hartl, 1993). A strong antibody response to GroEL has been found in cystic fibrosis patients with chronic pulmonary infection caused by P. aeruginosa (Ulanova et al., 1997), which suggests a role for PhrS under these conditions. The outer membrane porin OprD has been shown to facilitate diffusion of basic amino acids as well as of small peptides, and also serves as a channel for the β-lactam antibiotic imipenem (Trias & Nikaido 1990), whereby the loss of OprD can lead to imipenem resistance (Lynch et al., 1987). The proteome analysis upon overproduction of PhrS suggests that the sRNA could function as a riboregulator. Studies are under way to test whether PhrS regulates the identified protein genes in a direct or indirect manner.

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

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

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<th></th>
<th>MultiZ</th>
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<td>p ≥ 0.9</td>
<td></td>
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<tr>
<td>sRNAs RNomics‡</td>
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<td></td>
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</table>

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

### PAO1 sRNAs predicted by RNAz

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

Using NcDNAAlign alignments as input we found 115 structured candidate loci, of which 101 were previously
known and 14 are novel sRNA/ncRNA predictions. Based on the less restrictive MultiZ alignments, 221 candidates were predicted, of which 85 correspond to known sRNAs/ncRNAs. Details of the predicted loci are provided at http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/. The results of the RNAz screen for both MultiZ- and NcDNAlign-generated alignments are shown in Table 3. The number of RNAz hits that matched with sRNAs/ncRNAs predicted or experimentally tested by Livny et al. (2006) and Gonzalez et al. (2008) are indicated in Table 3. Information on these matches can be found at http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/. MultiZ-based alignments result in significantly more RNAz hits, both at high and low

Fig. 4. Steady-state levels of PhrD (a) and PhrS (b) in the presence (wt) or absence of Hfq (hfq−) at OD600 2 upon growth in LB medium and after serum exposure (Serum; see text). (c) Stability of PhrD (triangles) and PhrS (squares) in the presence (PAO1; closed symbols) and absence of Hfq (PAO1/hfq−; open symbols). Strains PAO1 and PAO1/hfq− were grown in LB medium to OD600 2.0. Total RNA was purified 0, 5, 15, 30 and 45 min after addition of rifampicin (100 μg ml−1 final concentration). The signals for the sRNAs were normalized to the respective signals of the 5S rRNA (loading control). The experiment was done in duplicate. Error bars, SD. The half-lives determined are given below the graph.

Fig. 5. (a) Comparison of the expression of plasmid-encoded phrS (a, lanes 2, 4 and 6; pMEphrS) and chromosomally encoded phrS (a, lanes 1, 3 and 5; pME4510). Total RNA was extracted from cells during exponential growth (OD600 = 0.8; lanes 1 and 2), upon growth to early stationary phase (OD600 = 1.5; lanes 3 and 4) and to stationary phase (OD600 = 2.5; lanes 5 and 6) in LB medium. In pMEphrS, the phrS gene was cloned under the transcriptional control of its authentic promoter (see Fig. 1e). (b) Identification of putative targets of PhrS by proteomics. The protein pattern of PAO1(pME4510) (left-hand panels) was compared to that of PAO1 harbouring pMEphrS (right-hand panels). The protein samples were taken in early stationary phase. Selected prominent spots of different intensity (black arrows) were identified by MS. Putative targets of PhrS were identified as GroEL, PA5153 and OprD. Only the relevant sections of the gels are shown.
Table 4. Novel RNAs predicted by both the MultiZ-based and the NcDNAAlign-based runs

<table>
<thead>
<tr>
<th>Locus*</th>
<th>p†</th>
<th>Locus*</th>
<th>p†</th>
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<tr>
<td>115</td>
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*Information on the sRNAs/ncRNAs encoded by the different loci is available at http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-023/. The loci in each row encode the same putative sRNA/ncRNA.

†Prediction confidence; see Table 3.

‡Expression of these loci, i.e. RNAs, has been verified by RT-PCR.

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

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

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