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The PqsR and RhlR transcriptional regulators determine the level of PQS synthesis in *Pseudomonas aeruginosa* by producing two different pqsABCDE mRNA isoforms

Stephan Brouwer¹, Christian Pustelny¹², Christiane Ritter³, Birgit Klinkert⁴, Franz Narberhaus⁴, Susanne Häussler¹²*  

**Affiliations:**  
¹Department of Molecular Bacteriology, Helmholtz Center for Infection Research, Braunschweig, Germany  
²Institute for Molecular Bacteriology, TWINCORE GmbH, Center of Clinical and Experimental Infection Research, a joint venture of the Hannover Medical School and the Helmholtz Center for Infection Research, Hannover, Germany  
³Department of Macromolecular Interactions, Helmholtz Center for Infection Research, Braunschweig, Germany  
⁴Microbial Biology, Ruhr University Bochum, Bochum, Germany

*Corresponding author:*  
Susanne Häussler  
Helmholtz Center for Infection Research, Inhoffenstraße 7,  
38124 Braunschweig, Germany  
[Susanne.Haeussler@helmholtz-hzi.de](mailto:Susanne.Haeussler@helmholtz-hzi.de)  
+49531-6181-3000

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Regulation of gene expression plays a key role in bacterial adaptability to changes in the environment. An integral part of this gene regulatory network is achieved via quorum sensing (QS) systems that coordinate bacterial responses under high cellular densities. In the nosocomial pathogen *Pseudomonas aeruginosa* the 2-alkyl-4-quinolone (*pqs*) signaling pathway is crucial for bacterial survival under stressful conditions. Biosynthesis of the *Pseudomonas* Quinolone Signal (PQS) is dependent on the *pqsABCDE* operon, which is positively regulated by the LysR-family regulator PqsR and repressed by the transcriptional regulator protein RhlR. However, the molecular mechanisms underlying this inhibition have remained elusive. Here, we demonstrate that not only PqsR but also RhlR activates transcription of *pqsA*. The latter uses an alternative transcriptional start site and induces expression of a longer transcript that forms a secondary structure in the 5’-untranslated leader region. As a consequence access of the ribosome to the Shine-Dalgarno sequence is restricted and translation efficiency reduced. Our finding of a competition between RhlR and PqsR for transcription initiation of the *pqsA-E* operon underlines that *P. aeruginosa* uses various levels of regulation to fine-tune PQS synthesis.
Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* is a ubiquitous bacterium which is able to thrive in a wide variety of environments. It is frequently associated with severe nosocomial infections and is found to be the leading cause of morbidity and mortality among people with cystic fibrosis (1). The remarkable ecological success of *P. aeruginosa* can be attributed to its large metabolic versatility and its sophisticated quorum sensing (QS) network. This cell-to-cell communication enables *P. aeruginosa* to control expression of numerous virulence factors and is involved in biofilm formation, thus facilitating establishment of acute and chronic infections.

QS in *P. aeruginosa* is tightly regulated by at least three different systems organized in a hierarchical manner. The AHL-dependent *las* system is considered to stand at the top of the hierarchy. It is composed of the LuxRI homologues LasR and LasI. The signal synthase LasI directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which is the ligand of the LasR receptor (2, 3). The *las* system is interconnected with the *rhl* system as LasR bound to its autoinducer 3-oxo-C12-HSL induces expression of RhlRI (4). LasR-3-oxo-C12-HSL and RhlR-C4-HSL direct transcription of several QS-regulated virulence factors, including pyocyanin, hydrogen cyanide and exotoxin A (5, 6). In addition, *P. aeruginosa* possesses a third QS system, *pqs*, based on 2-alkyl-4-quinolone (AQ) signal molecules (7). The *Pseudomonas* Quinolone Signal (PQS) and its direct precursor 2-heptyl-4-quinolone (HHQ) are active members of the over 50 different AQS produced by *P. aeruginosa* (8). Like AHLs, they play an important role in the expression of several virulence factors as well as in inducing a protective stress response towards deteriorating environmental conditions (9–11). Investigations of PQS biosynthesis revealed that the major synthase genes are arranged in a polycistronic operon (*pqsABCDE*) and that transcription of this operon is under control of PqsR, the Lys-R type transcriptional regulator of the *pqs* system (12, 13). PqsR, which is activated by PQS binding (14), plays a critical role in the pathogenicity of *P. aeruginosa* and is regulated by both the *las* and *rhl* system (13, 14) and the small RNA PhrS (15). While LasR activates *pqsR* transcription and subsequently enhances *pqsA-E* expression, RhlR was found to be a repressor of *pqsR* transcription (14). Interestingly, RhlR was also found to inhibit *pqsA-E* expression by binding to a *las/rhl* box centred at -311 bp upstream of the *pqsA* transcriptional initiation site (16). Recently, we could show that there is an alternative transcriptional start site (*pqsA*-339) just downstream of the RhlR-binding site (17), indicating that repression of PqsA expression via RhlR might be posttranscriptional.
Posttranscriptional repression events in prokaryotes mainly occur either by binding of small regulatory RNAs to target mRNA molecules or by formation of secondary structures in the mRNA, which play an important role in posttranscriptional regulation of gene expression in bacteria (18, 19). One common form of an RNA regulatory element is the so called riboswitch. These regulatory elements usually reside in the non-coding region of the mRNA and regulate gene expression by forming alternative structures in response to binding of a specific metabolite (20). Bacteria commonly mask the Shine-Dalgarno (SD) sequence to block access of the 30S ribosomal subunit. With this, translation initiation becomes highly dependent on the folding structure of the initiation region of the mRNA (21). A well-studied example of such a regulatory mechanism includes RNA thermosensors. They form a zipper-like structure which unwinds with increasing temperature allowing successful binding of the ribosome (22). For example, in Yersinia the virulence factor LcrF is expressed at 37°C, but access to the SD sequence is abolished at 26°C (23, 24). Thermosensors are also known to occur in Pseudomonas species. Recently, the bacterial small heat shock protein IbpA was found to be under control of two temperature-sensitive hairpin structures in the 5’-UTR of ibpA in P. aeruginosa (25).

In the present study, we show that repression of PQS biosynthesis by RhlR in P. aeruginosa is due to conformational masking of the translation initiation site of the pqsA-E transcript. We demonstrate that RhlR promotes transcription of pqsA-E from an alternative transcriptional start site. The resulting long 5’-UTR folds into a structure, which hinders association of the 30S ribosomal subunit with the mRNA and impedes translation initiation.
Results

RhIR induces transcription of the PQS biosynthetic operon via an alternative transcriptional start site

In an early attempt to investigate regulation of PQS synthesis, RhIR-C4-HSL was found to negatively regulate pqsR transcription and thus to inhibit PQS synthesis (14). Later, Xiao and colleagues showed binding of RhIR to a las/rhl box in the 5'-leader sequence of pqsA (Fig.1A) (15). Deletion of the entire las/rhl box significantly increased pqsA transcription in P. aeruginosa wild-type but not in rhIR mutant cells. It was therefore suggested that binding of RhIR to the las/rhl box lowers pqsA transcription (16).

To address this further, we generated luxCDABE promoter fusions that contained the recently predicted pqsA-339 (17) (p339), and the pqsA-71 (p71) transcription initiation site of the pqsA-E operon respectively (Fig.1B,C). Expression of lux in the P. aeruginosa PA14 parental strain and rhIR and pqsR mutant strains was monitored during exponential growth phase where the promoters have been predicted to be active (17). Consistent with Dötsch and colleagues (17), we found expression from the region containing pqsA-339 (Fig.2). RhIR acts as transcriptional activator of pqsA-339 and not as a repressor of pqsA transcription. While both promoters were active in the parental strain, p71 luminescence was only detected in PA14_pqsR while absent in PA14_rhIR. Vice versa pqsA-71 was only active in PA14_rhIR but silent in the absence of PqsR, demonstrating that induction of pqsA-339 is strictly dependent on RhIR.

The RhIR-dependent long 5'-UTR of pqsA blocks the translation initiation site

Since RhIR is an activator of pqsA transcription but represses PQS production, we hypothesized that base-pairing in the translation initiation region of the longer transcript might result in gene silencing due to inaccessibility of the SD sequence to the 30S ribosomal subunit. To test this hypothesis, we predicted the secondary structures of the pqsA-71 induced 5'-leader sequence (Fig.3A, short) and of a longer construct containing six additional nucleotides present in the pqsA-339 transcript (Fig.3A, long). In contrast to the short mRNA the long RhIR-induced transcript is predicted to sequester the SD sequence.

We next analyzed the thermal stability of 71–mer single-stranded DNA oligonucleotides equivalent to the sequence of the mRNAs (Fig.3B). To equate the expansion at the 5'-end of
the ‘long’ RhlR-induced primer (which comprises the entire sequence predicted to participate in hairpin formation), six nucleotides (TTCTGT) were added to the 3’-end of the ‘short’ primer. Both unfolding curves can be best explained by two separate unfolding events. Strikingly, the ‘long’ primer underwent thermal unfolding at significantly higher temperatures (44°C and 46°C) and with greater cooperativity than the ‘short’ oligonucleotide (26°C and 44°C). This strongly supports formation of a stable secondary structure in the RhlR-mediated transcript of pqsA-E. This effect could be reversed by site-specific mutagenesis of five nucleotides upstream of pqsA-71, responsible for base pairing with the SD region, with random non-complementary nucleotides (CGTTTC replaced by AAGAA).

To examine a potential inhibitory role of the secondary structure in the 5’-UTR of pqsA on translation efficiency, we generated additional promoter-fusion constructs (Fig.1C). The construct pshort contains both transcriptional start sites. plong harbors additionally the 70 nucleotides downstream of pqsA-71 containing the authentic RBS. Intriguingly, the presence of the 70 nucleotides significantly reduced (p < 0.05) luminescence in the wild-type strain and completely abolished luminescence in PA14_pqsR (Fig.4). In contrast, PqsR-dependent transcription in the rhlR mutant fully recovered luminescence and both constructs displayed similar activity. Interestingly, activation of pqsA-71 by PqsR in pshort was significantly enhanced in the absence of RhlR as compared to the wild-type (p < 0.01). The present data support our hypothesis, that RhlR mediates posttranscriptional control on pqsA-E expression by extending the 5’-UTR of pqsA that leads to alterations in the folding pattern of the mRNA in a way that inhibits efficient translation.

RhlR-induced transcription of the pqs operon abolishes efficient translation of the mRNA

Our data imply that formation of a secondary structure within the RhlR-induced long pqsA transcript inhibits pqsA translation. This effect should be reversed by site-specific mutagenesis liberating the SD sequence as depicted in Fig.3A. To test this, we analyzed PqsA expression in vivo using his-tagged pqsA whose expression was under control of the lac promoter but dependent on accessibility of the native SD sequence. Levels of PqsA-His were monitored during exponential growth in both P. aeruginosa PA14 and E. coli BL21 (Fig.5A). Strikingly, we were unable to detect PqsA-His in cells harboring the native RhlR-induced sequence while secondary structure destabilization in the derepressed construct drastically
restored PqsA production in both bacterial strains indicating that it is a general phenomenon not dependent on a *Pseudomonas*-specific factor.

To further assess the exact molecular role of the hairpin loop we performed primer extension inhibition (toeprinting) experiments to examine binding of the 30S ribosomal subunit to an mRNA molecule. In this method, ribosome-mRNA complex formation inhibits the primer extension reaction resulting in a terminated product (toeprint) around position +17 with respect to the translational start site. We used the assay to investigate the ability of the ribosome to recognize the SD sequence and to form a translation initiation complex upstream of *pqsA*. Efficiency of ribosome binding was compared between the following *in vitro* transcribed mRNAs: (1) PqsR-induced *pqsA* transcript, (2) RhlR-induced *pqsA* transcript, and (3) a mutated RhlR-induced mRNA exhibiting a destabilized secondary structure (Fig.5B).

Consistent with impaired translation from the long transcript, a toeprint at position +17 was detectable in the PqsR-induced *pqsA* transcript but absent in the RhlR-induced *pqsA* transcript (Fig.5C). Binding of the ribosome was re-established upon destabilization of the secondary structure. This clearly demonstrates that the secondary structure of the RhlR-induced *pqsA* transcript, which comprises the SD sequence, prevents formation of the pre-initiation complex. Additional reverse transcription products are indicative of additional double-stranded regions able to terminate reverse transcription (structures 1 and 2 in Fig.5C). Together these data demonstrate that RhlR mediates post-transcriptional repression of PQS synthesis by initiating a long *pqsA* transcript, in which the SD sequence is sequestered in a secondary structure.

**Discussion**

Pathogens have developed mechanisms to persist and survive in various environments including the human host. In *P. aeruginosa*, the production of the inter-bacterial signal molecule PQS is critical for survival under deteriorating conditions. PQS itself is a multifunctional molecule acting as a QS signal molecule (26), it has an iron-chelating activity and is essential for biofilm formation (6, 10). Furthermore, PQS plays a pivotal role in tuning cellular physiology and has been implicated in cell death under stressful conditions (6, 27). Recently, PQS was suggested to act as both a pro-oxidant and an inducer of an anti-oxidative stress response (9), emphasizing the importance of this molecule in environmental adaptation of *P. aeruginosa*. Therefore, expression of PQS needs to be strictly controlled.
The complex regulatory circuit of PQS synthesis involves the LysR-type transcriptional regulator protein PqsR, which recognizes and binds the signal molecule PQS and subsequently enhances transcription of the 4-quinolone biosynthetic operon pqsA-D thus forming a positive autoregulatory loop (14). Expression of pqsR in turn is controlled by the las and rhl QS systems, interconnecting all three QS systems of P. aeruginosa. However, the transcriptional regulator of the rhl system, RhlR, was also shown to directly exert control on PQS biosynthesis by binding to the pqsA-E promoter region (16). The present study provides molecular insights into this mechanism.

The discovery of an alternative transcriptional start site (pqsA-339) of the pqsA-E operon suggested an additional level of direct transcriptional regulation of the PQS system in P. aeruginosa (17). The fact that a RhlR-binding box is located just upstream of this transcriptional start site lead us to hypothesize that RhlR might be the transcriptional regulator of this promoter. Indeed, in the present study we demonstrate that RhlR is actually a transcriptional activator of pqsA-E and initiates transcription from pqsA-339. Further analyses revealed that RhlR binding to the pqsA promoter region induces the transcription of a pqsA-E mRNA with an extended 5'-UTR that exhibits a stable secondary structure, which sequesters the SD sequence and inhibits translation initiation of pqsA. As a consequence, a PA14_pqsR mutant displayed transcriptional activation of pqsA-339 but failed to efficiently induce production of PqsA. These data clearly show that RhlR competes with PqsR for binding to the promoter region of pqsA (Fig.6). By inducing a longer pqsA-E transcript, RhlR prevents translation of the pqsA gene, whose product is responsible for priming anthranilate for entry into the PQS biosynthetic pathway and whose deletion is known to impede PQS production (28, 29). Hence, depending on the presence of sufficient intra- and extracellular concentrations of the signal molecules C4-HSL and/or PQS, the PQS signaling pathway can either be induced by PqsR or inhibited by RhlR. The control of PQS expression by two transcriptional regulators of different QS systems thus allows P. aeruginosa to fine-tune PQS signaling in response to cell density and environmental stimuli.

Many bacterial mRNAs have been described to harbor structured elements in their 5'-leader sequence that control translation (30). Here, to our knowledge, we report for the first time on the modulation of bacterial protein levels via the production of two mRNA isoforms. Those isoforms form variable secondary structures and thus are translated at variable efficiency. We found that the competition between two transcription factors for transcription initiation result in alternative transcripts that have profound effects on cell-signaling dependent phenotypes.
Although not previously described in bacteria, different mRNA isoforms are well-known to play an important role in the regulation of translation in eukaryotes. In mammals up to 10-18% of all genes use multiple promoters (31). For instance, the axin2 gene, which is involved in early post-natal development and tumor suppression, has three promoters whose expression is strictly tissue-specific (32, 33). The 5´-UTR of each mRNA isoform affects translation efficiency and mRNA stability due to the formation of different secondary structures. Another example in eukaryotes is the tumor suppressor gene brca1 where transcription is induced from two separate promoters. Here, as observed for pqsA mRNA translation, a longer 5´-UTR of the brca1 mRNA is translated at lower efficiency due to formation of a stable secondary structure (34). Taken together, this study sheds light on the posttranscriptional regulation of PQS synthesis and illustrates that the promoter region of pqsA represents a major site of transcriptional and translational control. Differential secondary structures in mRNA isoforms that directly impact on translational efficiency of genes - a common mechanism of regulation in eukaryotes - might represent an underestimated mechanism of posttranscriptional control in bacteria and might play a more important role in bacterial adaptation than previously anticipated.
Materials and Methods

Bacterial strains and growth conditions

Unless otherwise noted, bacterial strains listed in Table 1 were grown in Luria broth (LB) medium at 37°C and shaking at 180 rpm. E. coli DH5α was routinely used for subcloning and propagation. For plasmid selection and maintenance, antibiotics were added at the following final concentrations (mg/ml): for E. coli, ampicillin 100; tetracycline 12.5; gentamicin 15; for P. aeruginosa, carbenicillin 400; tetracycline 100; gentamicin 30.

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant feature(s)</th>
<th>Source or reference</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>E. coli DH5α</td>
<td>F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Ph80dlacZAM15 Δ(lacZYA-argF) U169, hsdR17 (rK&lt;sup&gt;−&lt;/sup&gt; mK&lt;sup&gt;−&lt;/sup&gt;), h- F- ompT hsdS&lt;sub&gt;B&lt;/sub&gt; (r&lt;sup&gt;B&lt;/sup&gt; m&lt;sup&gt;B&lt;/sup&gt;) gal dcm</td>
<td>(35)</td>
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<tr>
<td>E. coli BL21 (DE3)</td>
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<td></td>
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<tr>
<td>E. coli S17-1</td>
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<td></td>
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<tr>
<td>PA14</td>
<td>Wild-type</td>
<td>Stratagene</td>
</tr>
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<td>PA14_&lt;sup&gt;pqsR&lt;/sup&gt;</td>
<td>pqsR knockout mutant</td>
<td>This study</td>
</tr>
<tr>
<td>PA14_&lt;sup&gt;rhlR&lt;/sup&gt;</td>
<td>rhlR knockout mutant</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pEX18Ap</td>
<td>Gene replacement vector with MCS from pUC18, oriT&lt;sup&gt;+&lt;/sup&gt; sacB&lt;sup&gt;+&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(37)</td>
</tr>
<tr>
<td>pEX18Ap2</td>
<td>pEX18TAp derivative, 845bp fragment containing 5S rRNA and lacZ-alpha genes and MCS removed by inverse PCR, novel MCS generated with unique restriction sites for XhoI, PstI, Smal/Xmal, XbaI, SacI, HindIII, NheI, NotI, MluI, KpnI, BamHI, EcoRI, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(38)</td>
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<tr>
<td>pEX18Ap-ΔpqsR::FRT-Gm</td>
<td>Gene replacement vector for PA14 pqsR containing a FRT-Gm cassette, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pEX18Ap2-ΔrhlR::FRT-Gm</td>
<td>Gene replacement vector for PA14 rhlR containing a FRT-Gm cassette, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pFLP3</td>
<td>FLP expression vector, sacB&lt;sup&gt;+&lt;/sup&gt; oriT&lt;sup&gt;+&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(39)</td>
</tr>
<tr>
<td>pBBR1-MCS5-Terminator-RBS-Lux (pMTRL)</td>
<td>Broad-host-range low-copy-number vector pBBR1-MCS5 harboring lacCDABE and terminators lambda T0 rmB1 T1 for plasmid-based transcriptional fusions, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(40)</td>
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<td>p339</td>
<td>-501 to -338 fragment upstream of pqsA cloned into pMTRL</td>
<td>This study</td>
</tr>
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<td>plasmid</td>
<td>description</td>
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<tr>
<td>p71</td>
<td>using SpeI and PstI sites, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pshort</td>
<td>-256 to -70 fragment upstream of (pq) cloned into pMTRL using SpeI and PstI sites, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>plong</td>
<td>-501 to +1 fragment upstream of (pq) cloned into pMTRL using SpeI and PstI sites, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pUCP20</td>
<td>Escherichia-Pseudomonas shuttle vector with beta-lactamase ((bla)) and LacZ alpha peptide ((lacZ\alpha)) genes; Ap&lt;sup&gt;r&lt;/sup&gt;/Cb&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(\text{(41)})</td>
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<td>pUCP20-TOEpqA&lt;sub&gt;(1)&lt;/sub&gt;</td>
<td>-72 to +60 fragment of (pq) containing a T7 promoter (5'-GAA ATTAATACGACTCACTATAGG-3') and a EcoRI restriction site at the 3'-end cloned into pUCP20 using the Smal site, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pUCP20-TOEpqA&lt;sub&gt;(2)&lt;/sub&gt;</td>
<td>-140 to +60 fragment of (pq) containing a T7 promoter (5'-GAA ATTAATACGACTCACTATAGG-3') and a EcoRI restriction site at the 3'-end cloned into pUCP20 using the Smal site, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pUCP20-TOEpqA&lt;sub&gt;(3)&lt;/sub&gt;</td>
<td>pUCP20-TOEpqA&lt;sub&gt;long&lt;/sub&gt; containing a mutation in the 5'-UTR of (pq) where CGTTC was replaced by AAGAA, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pUCP20_&lt;sup&gt;Δ&lt;/sup&gt;RBS</td>
<td>Plasmid-borne ribosomal binding site AGGAAA of pUCP20 was replaced by CCTCGC; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pUCP20_&lt;sup&gt;Δ&lt;/sup&gt;RBS-pqA-His6</td>
<td>Fragment containing -80 bp of the 5'-UTR and the entire coding sequence of (pq) was cloned into pUCP20_&lt;sup&gt;Δ&lt;/sup&gt;RBS using KpnI and HindIII sites, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pUCP20_&lt;sup&gt;Δ&lt;/sup&gt;RBS-pqA-His6</td>
<td>pUCP20_&lt;sup&gt;Δ&lt;/sup&gt;RBS-pqA-His6 containing a mutation in the 5'-UTR of (pq) where CGTTC was replaced by AAGAA, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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### Construction of knockout mutants

To create the single knockout mutants in the wild-type PA14 parental strain we used an adapted version of the gene replacement method by (37) with use of plasmid pEX18Ap for \(pq\) and pEX18Ap2 (38) for \(rh\), and a gentamicin resistance cassette flanked by Flippase Recombination Target (FRT-Gm) originating from plasmid pPS856. The mutant fragments were constructed by PCR extension overlap (42) with the following primers for \(pq\): upstream region \(pq\)/\(pq\)/\(pq\)/\(pq\), downstream region \(pq\)/\(pq\)/\(pq\)/\(pq\), and for \(rh\): upstream region \(rh\)/\(rh\)/\(rh\)/\(rh\), downstream region \(rh\)/\(rh\)/\(rh\)/\(rh\) (Table 2). The BamHI site was introduced between the upstream and downstream region of \(pq\) and the NheI site for \(rh\) respectively. These restriction sites were used to insert the FRT-Gm cassette. In case of \(pq\), the primers were designed to target for deletion the 529bp upstream region of \(pq\), which comprise of all promoter binding sites, as well as 487bp part of the coding sequence, while for \(rh\) the primers were designed to delete the entire coding sequence of the gene. The resulting
plasmids pEX18Ap-ΔpqsR::FRT-Gm and pEX18Ap2-ΔrhlR::FRT-Gm were transferred into
P. aeruginosa PA14 by two-parental mating using the donor strain E. coli S17-1. P. aeruginosa
cells were selected on nalidixic acid (20μg/ml) and gentamicin (50μg/ml). The
occurrence of the double cross-over was checked by plating at least 30 colonies from the
mating result on gentamicin and carbenicillin (400μg/ml) containing agar plates. Gentamicin
resistant and carbenicillin sensitive bacteria were isolated and the insertion of the FRT-Gm
cassette ensured by PCR. Finally the FRT-Gm cassette was removed from the chromosomal
dNA with help of flipase encoded on pFLP3 plasmid (39). The knockout mutation was
confirmed by PCR using primers annealing outside of any pEX18Ap/pEX18Ap2 mediated
deletion regions.

**Bioluminescence assays**

To generate promoter-*luxCDABE* (*lux*) fusions, *pqsA* promoter fragments were PCR-
amplified from PA14 chromosomal DNA, digested with SpeI and PstI, and subcloned into
pMTRL (40). P339 was generated from primers *pqsA*-339-SpeI-fw/*pqsA*-339-PstI-rv, p71
from primers *pqsA*-71-SpeI-fw/*pqsA*-71-PstI-rv, pshort from primers *pqsA*-339-SpeI-
fw/*pqsA*-71-PstI-rv, and plong from primers *pqsA*-339-SpeI-fw/*pqsA*-ATG-PstI-rv (Table 2).
The resulting plasmids were transferred into the *P. aeruginosa* strains PA14 wild-type,
PA14_ΔpqsR and PA14_ΔrhlR. The transcriptional reporter strains were grown overnight with
the appropriate antibiotic, then subcultured from an OD <sub>600</sub> of 0.05 in BM2 medium [7 mM
(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 40 mM K<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 0.4% glucose and 2 mM MgSO<sub>4</sub>] containing
0.01% CAS amino acids and 10 μM FeSO<sub>4</sub>, and grown at 37°C. Bioluminescence was
monitored using an EnSpire Multimode Plate Reader (PerkinElmer). Promoter activities are
given as the relative luminescence of 200 μl of the cultures measured in a 96-well plate
divided by the OD<sub>600</sub> (relative light units [RLU] OD<sub>600</sub>⁻¹). All results represent the mean of at
least three independent replicates.

**Thermal unfolding of DNA oligonucleotides**

UV absorption spectra of 71 bp-long oligonucleotides (Table 2) were recorded on a JASCO J-
815 CD spectrometer at a concentration of 10 μM in 50 mM potassium phosphate buffer pH
7.2. Thermal unfolding of secondary structure was monitored as an increase in absorption at
255 nm as a function of temperature in intervals of 1°C and a ramp rate of 4°C per minute. To
determine the melting temperatures, raw data were fitted with an equation for a dual step
unfolding of a monomer with corrections for linear changes of the CD signal before and after
the unfolding transition (43).

**Generation of His6-tagged fusion *pqsA* and Immunoblotting**

The impact of folding structures in the 5’-UTR on the in vivo translation efficiency of *pqsA* in
*E. coli* and *P. aeruginosa* was analyzed by cloning C-terminal his-tagged *pqsA* from
nucleotide -80 to the stop codon into the KpnI/HindIII sites of pUCP20 ΔRBS, a pUCP20
derivate lacking the plasmid-borne ribosomal binding site generated by site-specific
mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies) using the
primer set pUCP20_RBSmut-fw/pUCP20_RBSmut-rv (Table 2), according to the
manufacturer's instructions. pUCP20 ΔRBS-*pqsA*-His6 was generated using the primer set
*pqsA* KpnI-fw/*pqsA* _6His-rv and primers *pqsA* _mut_KpnI-fw/*pqsA* _6His-rv were used for
generation of pUCP20 ΔRBS-*ΔpqsA*-His6 (Table 2). To prepare samples for Western Blot
analysis, whole-cell lysates of cultures grown to exponential growth phase in LB medium
were normalized for protein content and 10 µl of an OD<sub>600</sub> of 10.0 were separated by SDS-
PAGE (10% acrylamide) after 15 min incubation at 95°C. As primary antibody we used a
His-Tag mouse IgG<sub>1</sub> monoclonal antibody (Novagen) at a dilution of 1:1000. A4a goat anti-
mouse IgG & IgM (Dianova) was used as secondary antibody at a dilution of 1:2000. Blots
were developed using Lumi-Light Western Blotting Substrate (Roche) and
chemiluminescence was detected using a Las-1000 Luminscent Image Analyzer (Fujifilm).

**Toeprinting analysis**

Primer extension inhibition (toeprinting) assays were performed as described previously (24).
Plasmid templates were generated by cloning PCR-amplified fragments comprising a T7
promoter sequence (5’-GAAATTAATACGACTCACTATAGG-3’) at the 5’-end, a EcoRV
site at the 3’-end, and -140 (2) and -72 (1) to +60 nucleotides of *pqsA*, with usage of primers
T7-*pqsA*200nt-fw/Toe_EcoRV-rv and T7-*pqsA*132nt-fw/Toe_EcoRV-rv (Table 2), into SmaI
cut pUCP20 (blunt-end treated) resulting in pUCP20-TOE*pqsA* (2) and pUCP20-
TOE*pqsA* (1), respectively. To generate pUCP20-TOE*pqsA* (3) site-specific mutagenesis of
pUCP20-TOE*pqsA* (2) was carried out using the QuikChange II Site-Directed Mutagenesis
Kit (Agilent Technologies) with the primers 5’UTRpqsA_mut-f/5’UTRpqsA_mut-r (Table 2),
according to the manufacturer's instructions. For in vitro transcription with T7 RNA
polymerase plasmids were linearized by digestion with EcoRV and the primer Toe_short-rv
was used for reverse transcription. The experiments were carried out at 37°C in the presence and absence of *E. coli* 30S ribosomal subunits.

Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’ direction)*</th>
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<tr>
<td><strong>Mutagenesis</strong></td>
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<tr>
<td>pqsRup2FEcoRI</td>
<td>GAGAATTCATCCACCGGGCAGCCCAG</td>
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<tr>
<td>pqsRup2RBamHI</td>
<td>CGGGATCCGTTAGCAGCCAACGGCCAGGC</td>
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<td>pqsRdwFcommpup</td>
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<td>pqsRdwR HindIII</td>
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<td>up/rlnot1-fw</td>
<td>TATGCGGCGCTGACACGGCTACGCG</td>
</tr>
<tr>
<td>up/rlnhel1-rv</td>
<td>TCAGTCAGTCGACCTAAGGAGGATCGGATAAAATGCA</td>
</tr>
<tr>
<td>dorh/lRnhe1-fw</td>
<td>GCTAGCTGACTGACGAAGCCAGGCGGC</td>
</tr>
<tr>
<td>dorh/lRnhe1-rv</td>
<td>TATAAGCTTGACGCGGTAGCGGAAGGC</td>
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<tr>
<td><strong>Promoter-lux fusions</strong></td>
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<td>pqsA-339-SpeI-fw</td>
<td>TCAGACTAGTGAGGCTGCAATGGCA</td>
</tr>
<tr>
<td>pqsA-339-PstI-rv</td>
<td>ATACTGCAGGCAAAAACATGACAAACCCACCTTG</td>
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<tr>
<td>pqsA-71-SpeI-fw</td>
<td>TCAGACTAGTGACCCCTTCCTTG</td>
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<td>pqsA-71-PstI-rv</td>
<td>ATACTGCAGGGAACGGAGACCGG</td>
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<tr>
<td>pqsA-ATG-PstI-rv</td>
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<td><strong>CD Spectroscopy</strong></td>
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<td>long</td>
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<tr>
<td>derep</td>
<td>AAAGACTGACCAAAAGCAGACCCCTCAGGGGTATCTCCTGTACCGGAGGAGGAGG</td>
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<td><strong>His-tagged pqsA</strong></td>
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<tr>
<td>pUCP20_RBSmut-fw</td>
<td>GGAATTGTGATCGGATAACACCTTCAACCACCGTCAGTGCATAGCATGATTAC</td>
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<td>pUCP20_RBSmut-rv</td>
<td>GAAAGGCGAGAAGGAGGAGGAGGAGG</td>
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<tr>
<td>pqsA_KpnI-fw</td>
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<td>TATCGGTACCACCCCAAGAAGCAGCAGGAGGGGAGGAGGAGGAGGAGG</td>
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<td>pqsA_6His-rv</td>
<td>TATGAACCTTCATCACGTGGCTGAGGTGATGGTAGGATGTGAGGAGGAGGAGGAGGAGG</td>
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<tr>
<td><strong>Toeprinting</strong></td>
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Acknowledgments

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References


Figure legends

**Fig.1:** Regulatory elements within the 5'-UTR of the *pqsA* gene and constructs used in this study. (A) Promoter region of *pqsA*. The *pqsA* transcriptional start sites are indicated by bent arrows at *pqsA*-339 and *pqsA*-71, and the *pqsA* ATG start codon is underlined. The binding sites of RhlR, PqsR and the ribosome are boxed and labeled. (B) Model of the *pqsA* promoter region. (C) *pqsA* promoter-fusion constructs used in the present study. (-) and (+) indicate position to the start codon of *pqsA*. Lines are not drawn to scale.

**Fig.2:** Promoter activity of the two alternative transcriptional start sites of *pqsA*. *P. aeruginosa* strains PA14 WT, *pqsR* and *rhlR* mutants containing plasmids p339 and p71 were cultured as described in Material and Methods and assayed for *lux* expression. Data are presented in relative luminescence and error bars represent one standard deviation of the mean value from three biological replicates (*p ≤ 0.05 and **p ≤ 0.01*).

**Fig.3:** Folding dynamics and stability of secondary structures formed by the 5-UTR of *pqsA*. (A) Predicted secondary structures in the 5'-leader sequence of the mRNA expressed by PqsR (short; -14.10 Kcal/mol), RhlR (long; -29.80 Kcal/mol) and a derepressed RNA variant (derep; -14.10 Kcal/mol). Secondary structures were generated using the CONTRAfold method (44). The color-code represents base-pairing probabilities in the structure ensemble, whereby high values (red) close to 1 are the most probable. Numbers indicate nucleotide positions relative to A of the AUG start codon. The Shine-Dalgarno sequence AGGGAA is encircled and mutated nucleotides are emphasized by an asterisk (*). (B) Thermal unfolding behavior of 71-mer single-stranded DNA oligonucleotides equivalent to the RNA sequences shown in (A) monitored by UV-absorption at 255 nm. Markers represent the raw data recalculated to the fraction of unfolded DNA. Continuous lines represent the data fits used to calculate the melting temperatures.

**Fig.4:** Inclusion of the SD sequence in the RhlR-induced *pqsA* mRNA inhibits translation. *P. aeruginosa* strains PA14 WT, *pqsR* and *rhlR* mutants containing plasmids pshort and plong were cultured as described in Material and Methods and assayed for *lux* expression. Data are presented in relative luminescence and error bars represent one standard deviation of the mean value from three biological replicates (*p ≤ 0.05 and **p ≤ 0.01*).

**Fig.5:** Translational control of *pqsA* expression. (A) Analysis of His6-tagged PqsA expressed under *lac* promoter control but in dependence of the native translation initiation site
in *E. coli* BL21 and PA14 WT. *In vivo* levels of PqsA-His were compared between the wild-type 5-UTR comprising -80 nucleotides relative to the start codon and a derepressed 5-UTR causing liberation of the SD sequence. (B) Predicted folding patterns of the *in vitro* transcribed *pqsA* constructs used for the toeprint assay: (1) PqsR-induced *pqsA* transcript (-22.70 Kcal/mol), (2) RhlR-induced *pqsA* transcript (-46.40 Kcal/mol), and (3) a mutated RhlR-induced mRNA exhibiting a destabilized secondary structure (-29.80 Kcal/mol). Folding dynamics of the mRNA constructs were predicted using the CONTRAfold method (44). Secondary structures are color-coded according to base-pairing probabilities in the structure ensemble, whereby high values (red) close to 1 are the most probable. The Shine-Dalgarno sequence AGGGAA is encircled and the AUG start codon is indicated by a black line. Site-specific mutated nucleotides are emphasized by an asterisk (*). (C) Primer extension inhibition assay of the *pqsA* mRNAs drawn in (B), including the 5'-UTR and the first 60 nucleotides of the *pqsA* coding sequence. Addition (+) or absence (-) of *E. coli* 30S ribosomal subunits is indicated. The AUG start codon and the SD sequence are marked on the right and full-length products, structure 1 and structure 2 and the toeprint are indicated on the left-hand side.

**Fig.6: Model of transcriptional and translational control of *pqsA***. *P. aeruginosa* releases the signal molecules N-butanoyl-L-homoserine lactone (C4-HSL) and the *Pseudomonas* quinolone signal (PQS) in a cell-dependent manner. Upon binding to their cognate ligand, the transcriptional regulators RhlR-C4-HSL and PqsR-PQS induce transcription of the *pqsA-E* operon from the transcription start sites *pqsA*-339 and *pqsA*-71, respectively. In the *pqsA*-339-induced mRNA the formation of a hairpin at the translation initiation site of *pqsA* blocks access of the ribosome to the SD-sequence. As a consequence, production of the anthranilate-coenzyme A ligase PqsA is hindered rendering *P. aeruginosa* unable to generate HHQ / PQS.
