RgsA, an RpoS-dependent sRNA, negatively regulates rpoS expression in Pseudomonas aeruginosa

Pei Lu, Yifei Wang, Yangbo Hu and Shiyun Chen*

Abstract
As a master regulator, the alternative sigma factor RpoS coordinates the transcription of genes associated with protection against environmental stresses in bacteria. In Pseudomonas aeruginosa, RpoS is also involved in quorum sensing and virulence. The cellular RpoS level is regulated at multiple levels, whereas the post-transcriptional regulation of rpoS in P. aeruginosa remains unclear. To identify and characterize small regulatory RNAs (sRNAs) regulating RpoS in P. aeruginosa, an sRNA library expressing a total of 263 sRNAs was constructed to examine their regulatory roles on rpoS expression. Our results demonstrate that rpoS expression is repressed by the RpoS-dependent sRNA RgsA at the post-transcriptional level. Unlike OxyS, an sRNA previously known to repress rpoS expression under oxidative stress in Escherichia coli, RgsA represses rpoS expression during the exponential phase. This repression requires the RNA chaperone Hfq. Furthermore, the 71–77 conserved region of RgsA is necessary for full repression of rpoS expression, and the –25 to +27 region of rpoS mRNA is sufficient for RgsA-mediated rpoS repression. Together, our results not only add RgsA to the RpoS regulatory circuits but also highlight the complexity of interplay between sRNAs and transcriptional regulators in bacteria.

INTRODUCTION
Small regulatory RNAs (sRNAs) are important post-transcriptional regulators of genes involved in many cellular processes and virulence in bacteria [1–3]. sRNAs perform regulatory roles either by base-pairing with target mRNAs to control premature transcription termination, modulate their translation initiation and/or mRNA stability, or by modulating protein activity upon interaction [1, 4–6]. In many bacteria, Hfq acts as an RNA chaperone, which affects sRNAs’ stability, and facilitates sRNAs’ base-pairing with their target mRNAs [5, 7].

As a sigma subunit of the RNA polymerase, RpoS is a master regulator important for cell adaptation to a wide array of stresses, including nutrient starvation and oxidative stress [8, 9]. In the opportunistic pathogen Pseudomonas aeruginosa, over 700 genes are under the control of RpoS [10]. Although RpoS contributes to the response to several environmental stresses in P. aeruginosa, it plays more specific roles on the transcription of genes related to virulence and quorum sensing (QS) [9–11].

In E. coli and Salmonella, the regulation of RpoS expression occurs mainly at the post-transcriptional level, depending on the secondary structure of its 5′ UTR (untranslated region), sRNAs and protein stability involving ClpXP protease [12, 13]. The long 5′ UTR of rpoS mRNA folds into a stem-loop secondary structure, which occludes the ribosome-binding site to block access of the 30S ribosome [14]. Three Hfq-binding sRNAs, DsrA, RprA and ArcZ, directly interact with the 5′ UTR of the rpoS mRNA to open the inhibitory hairpin, thereby releasing the ribosome-binding site [12, 13]. Meanwhile, these sRNAs also inhibit premature Rho-dependent transcription termination of rpoS mRNA [15]. Another Hfq-binding sRNA in E. coli, OxyS, reduces RpoS levels under oxidative stress, but its mechanism of action on rpoS is not fully understood [16, 17].

Previous studies indicated that translational regulation should also exist in Pseudomonas [18, 19]. It was revealed that the ribosomal protein S1 specifically binds to rpoS mRNA 5′ UTR during the exponential growth phase, whereas the regulatory effect on RpoS synthesis is currently unknown [18]. In addition, the secondary structure of the rpoS mRNA leader is involved in the translational regulation of rpoS expression in Pseudomonas putida [19]. Besides, some other trans-regulators, such as RNA-binding proteins and sRNAs, may also be responsible for rpoS expression in Pseudomonas, which need to be further investigated [19]. Moreover, Hfq is required for efficient
rpoS expression in P. aeruginosa PAO1 at the stationary phase [20]. However, no sRNA responsible for post-transcriptional regulation of rpoS in Pseudomonas has been identified.

Over 500 sRNAs have been identified in P. aeruginosa [21], but only a few have been further investigated. In this study, we constructed a sRNA overexpression library to identify sRNAs that affect the expression of a rpoS-lacZ post-transcriptional fusion. Our results identify RgsA as a negative regulator of rpoS expression in P. aeruginosa.

Originally named P16, RgsA is an sRNA conserved in Pseudomonas and has also been identified in Azotobacter vinelandii [22–24]. Its transcription is directly activated by RpoS and indirectly by the GacS/GacA two-component system [22]. RgsA has been shown to directly target the global transcriptional regulator fis and acyl carrier protein acpP mRNA [25]. It was also found that RgsA contributes to a number of physiological processes, including the oxidative stress response [22, 23, 26], pyocyanin production, motility and growth rate in the exponential phase [25], but in unknown regulatory circuits.

In this study, we provide evidence that RgsA decreases rpoS mRNA as well as the RpoS protein level in the exponential growth phase, and the inhibition is dependent on Hfq. In terms of biological significance, our results add RgsA to the number of connections between sRNAs and transcriptional regulators.

METHODS

Bacterial strains, mutant construction and growth conditions

Bacterial strains and plasmids used in this study are summarized in Table 1. The wild-type strain used in this study is Pseudomonas aeruginosa PAO1. A P. aeruginosa mutant was generated using the suicide plasmid pDM4 as described previously [25, 27]. Briefly, to obtain the ΔrpoS::kan mutant, an upstream fragment from +454 to +1 of rpoS (according to its translational start codon) and a downstream 517 bp fragment from the stop codon of rpoS were amplified from PAO1 genomic DNA with oligonucleotides PLO-241/PLO-326 and PLO-327/PLO-328 (Table S1, available in the online version of this article), respectively. PCR products were digested with specific restriction enzymes and then inserted into pDM4-Kan treated with the same enzymes. The conjugation was performed as described previously [25]. The mutant was confirmed by PCR with oligonucleotides PLO-241/PLO-328 (Table S1) and sequencing. For standard cultures, E. coli and P. aeruginosa strains were grown in Luria–Bertani (LB) medium at 37 °C. Where appropriate, antibiotics were used as follows: for E. coli, chloramphenicol 30 µg ml⁻¹, gentamicin 15 µg ml⁻¹, tetracycline 25 µg ml⁻¹ and kanamycin 100 µg ml⁻¹; and for P. aeruginosa, gentamicin 50 µg ml⁻¹, kanamycin 400 µg ml⁻¹, tetracycline 125 µg ml⁻¹ and streptomycin 100 µg ml⁻¹.

Construction of plasmids

Oligonucleotides used in this study are summarized in Table S1. For the sRNA library, the sRNA genes were amplified by PCR from the P. aeruginosa PAO1 genome using the oligonucleotides listed in Table S1. PCR products were treated with PstI/EcoRI or EcoT22I/EcoRI and inserted into the PstI/EcoRI-digested pROp backbone, as previously described [25]. Post-transcriptional rpoS-lacZ fusions were constructed using PCR products amplified from genomic DNA as described previously [25]. Inserts were treated with KpnI/PstI and ligated into an equally treated pME9533placO plasmid backbone. Details of the cloned inserts are summarized in Table 1. To construct transcriptional rpoS-lacZ fusion pME6522-rpoS, oligonucleotides PLO-457 and PLO-458 were used to amplify a 357 bp fragment from genomic DNA. PCR products were digested with EcoRI and PstI, and inserted into similarly treated pME6522.

Western blot analysis

Overnight culture of P. aeruginosa strains was diluted 1/200 into fresh LB medium. When cells were grown to the indicated OD₆₀₀, 1 ml of culture was collected and resuspended in sample loading buffer at a final concentration of 0.01 OD µl⁻¹. To analyse protein levels by Western blot, 0.1 OD cells per lane were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore). RpoS and RpoA protein levels were detected using an antiserum recognizing RpoS (1 : 2000; rabbit), an antiserum recognizing RpoA (1 : 2000; rabbit) and HRP-conjugated anti-rabbit secondary antibody.

RNA extraction and Northern blot analysis

Total RNA was isolated using the TRizol reagent (Invitrogen) following the manufacturer’s instructions. RNA samples (5 µg or 10 µg) were next analysed by Northern blot as described previously [25].

Real-time RT-PCR

Reverse transcription with a random primer was performed as described previously [25]. The first-strand cDNAs were produced by M-MLV Reverse Transcriptase (Promega) from DNase I (Promega) treated RNA samples (2 µg). The rpoS mRNA level was quantitatively assessed by real-time RT-PCR using the iTaq Universal SYBR Green Supermix (Bio-Rad). Oligonucleotides used were PLO-353/PLO-354 (rpoA), PLO-355/PLO-356 (rpoS).

β-galactosidase assay

Overnight cultures of P. aeruginosa strains were inoculated 1/200 into fresh LB medium. Cells were withdrawn at the indicated OD₆₀₀ and then the β-galactosidase activity was assessed by the Miller method [28]. All of the experiments were independently performed in triplicate.

Statistical analysis

Mean values were derived from the results of multiple independent tests. Error bars represented SD. Statistical analyses were done by Student’s t-test. A value of P<0.05 or P<0.005
was considered to be statistically significant (*), or significant (**), respectively.

**RESULTS**

**Screening the sRNA library for post-transcriptional regulation of an rpoS-lacZ fusion**

To identify and characterize sRNAs regulating RpoS in *P. aeruginosa*, a set of 263 sRNAs were cloned into the pROp plasmid [21], such that they were driven by the P\_P. aeruginosa promoter from their 5’ ends. Due to the fact that the vast majority of sRNA-mediated regulation takes place at the 5’ UTR and 5’ CDS (coding sequence) of the target genes, we constructed a plasmid-borne rpoS-lacZ post-transcriptional fusion spanning from the rpoS transcriptional start site to the 27th nucleotide of its coding region under the control of the P\_LlacO promoter [29]. Thus, expression of the rpoS-lacZ fusion should only be sensitive to the regulation of rpoS at the post-transcriptional level.

Each of the sRNA over-expressing plasmids in combination with the P\_LlacO-rpoS-lacZ fusion plasmid was transformed into *P. aeruginosa* PAO1 wild-type strain. These transformants were grown to an OD\_600 of 0.8, we monitored the β-galactosidase activity. Regulation by greater than two-fold was considered to be statistically significant (*), or significant (**), respectively.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant characteristics and synonym</th>
<th>Reference</th>
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<td><em>E. coli</em> TOP10</td>
<td>merA Δ(mcrAB-5Ru-3hsdMBSmR-gmr-2) Tn5 lacZΔM15 ΔargF769 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)F7697 galU galK rpsL endA1 supG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> S17-1</td>
<td>λ-pir lysogen of S17-1, thy hsdRS17 R1 pir relA1 galK supE44 thi pro hsdR hsdM15 recA1 araD139 Δ(ara-leu)F7697 galU galK rpsL endA1 supG</td>
<td>Lab collection</td>
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<td><em>P. aeruginosa</em> PAO1</td>
<td>Wild-type <em>P. aeruginosa</em> PAO1</td>
<td>[25]</td>
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<tr>
<td>ΔrpoS::kan</td>
<td>rpoS gene replaced by kanamycin cassette in PAO1, Kan^'</td>
<td>This study</td>
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<tr>
<td>Δhfq::Sm</td>
<td>PAO1 derivative with streptomycin cassette insertion into hfq coding region</td>
<td>[25]</td>
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<td>ΔrpoS::kan</td>
<td>rpoS gene replaced by kanamycin cassette in PAO1, Kan^'</td>
<td>This study</td>
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<td>pROp200</td>
<td>Control plasmid based on pBRR1 MCS-5, Gm^'</td>
<td>[25]</td>
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<td>pROp-RgsA</td>
<td>RgsA; <em>P. aeruginosa</em> PAO1 rpoS expression plasmid, controlled by the constitutive P_rpoS promoter, Gm^'</td>
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<td>RgsA_AT5-64; <em>P. aeruginosa</em> PAO1 rpoS_AT5-64 (deletion of position 58–64) expression plasmid, rgsA_AT5-64 is controlled by the constitutive P_rpoS promoter, Gm^'</td>
<td>This study</td>
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<td>pROp-RgsA_AT71-77</td>
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<td>pME9533_P_LlacO</td>
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<td>[25]</td>
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<td>pME6522</td>
<td>pVS1-p15A shuttle vector for constructing the transcriptional lacZ fusions, Tc^'</td>
<td>[43]</td>
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<td>pME9533_P_LlacO::rpoS1</td>
<td>rpoS1; rpoS-lacZ; expresses rpoS1(-366+27):lacZ translational fusion from constitutive P_LlacO promoter, Tc^'</td>
<td>This study</td>
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<td>pME9533_P_LlacO::rpoS2</td>
<td>rpoS2; expresses rpoS2(-191+27):lacZ translational fusion from constitutive P_LlacO promoter, Tc^'</td>
<td>This study</td>
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<td>pME9533_P_LlacO::rpoS4</td>
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<td>This study</td>
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<td>pME6522::rpoS</td>
<td>P_P. aeruginosa lacZ; rpoS transcriptional lacZ fusion plasmid, Tc^'</td>
<td>This study</td>
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<td>pDM4::Kan^'</td>
<td>The kanamycin marker was inserted into the multi-cloning sites of pDM4, Cm^', Kan^'</td>
<td>[25]</td>
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confirmed by Western blot analysis. As seen in Fig. 2(b), rgsA deletion modestly up-regulated RpoS protein level, while expression of RgsA from the pROp-RgsA plasmid reduced the RpoS level 2.4-fold in the exponential phase. However, RgsA poorly repressed RpoS expression in the stationary phase (Fig. 2b). Northern blot analysis showed that endogenous RgsA is transcribed since exponential phase and peaks in the stationary phase, and is absent in ΔrgsA::kan strain (Fig. 2b) [25]. High levels of RgsA were observed in the presence of RgsA overexpression plasmid (Fig. 2b).

We next tested whether RgsA controls the rpoS mRNA level by real time RT-PCR. As seen in Fig. 2(c), the rpoS mRNA level was also reduced upon RgsA overexpression at the exponential growth stage, while the repression was abrogated in cells grown into stationary phase. Meanwhile, we also examined the effect of constitutive RgsA expression on the transcriptional rpoS-lacZ fusion (Fig. 2d). The promoter cloned in this fusion encompassed the predicted −341 to +1 region relative to the rpoS transcription start site. Compared with the control vector, the expression of the transcriptional rpoS-lacZ fusion was not changed upon RgsA overexpression (Fig. 2d), suggesting that the rpoS mRNA level is reduced by RgsA at the post-transcriptional level. Collectively, these observations indicate that RgsA represses rpoS expression at the post-transcriptional level in exponentially growing cells.

**Hfq is required for RgsA down-regulation of rpoS expression**

The intracellular accumulation and function of many sRNAs require the RNA chaperone Hfq [1, 30]. Our previous study also revealed that Hfq is involved in RgsA accumulation as well as negative regulation of fis and acpP mRNA induced by RgsA [25]. As RgsA could interact with Hfq in P. aeruginosa [26, 31], we investigated whether Hfq
is involved in the efficient regulation of rpoS by RgsA. The effect of RgsA overexpression on rpoS expression was measured in P. aeruginosa PAO1 wild-type and Δhfq::Sm strains. As previously observed, RgsA inhibited RpoS expression in the wild-type strain, but no such effect was seen in Δhfq::Sm (Fig. 3a). Meanwhile, RgsA-dependent reduction of RpoS protein level in the wild-type strain was accompanied by a decrease in rpoS mRNA level, which was abolished in Δhfq::Sm (Fig. 3b).

We next examined the regulatory effect of RgsA on rpoS-lacZ post-transcriptional fusion in the wild-type and Δhfq::Sm strains. Consistent with the above results, RgsA reduced rpoS-lacZ expression in the exponentially growing wild-type strain, whereas hfq deletion abrogated the regulatory effect of RgsA (Fig. 3c, left panel). Upon cells grown into stationary phase, RgsA failed to regulate rpoS-lacZ expression in both wild-type and hfq deletion strain (Fig. 3c, right panel). Additionally, the β-galactosidase activity of rpoS-lacZ is lower in the Δhfq::Sm background both in the presence or absence of RgsA (Fig. 3c). Since transcription of the rpoS::lacZ mRNA is driven by the P_{LlacO} promoter that is not affected by Hfq, Hfq may contribute to rpoS::lacZ mRNA stability and/or its translation initiation directly or indirectly by a different sRNA. However, the absence of Hfq has no influence on chromosomally encoded RpoS protein and rpoS mRNA levels (Fig. 3a, b). This may be caused by overlaying transcriptional and post-translational controls. Taken together, these results indicate that Hfq is required in RgsA-mediated repression of rpoS expression.
The conserved region of RgsA mediates the repression of rpoS expression

RgsA is a conserved sRNA in Pseudomonas and directly regulates fis and acpP mRNA via a conserved single-stranded region [22, 25]. As previously reported, the 72–86 region of RgsA is highly conserved in all identified rgsA sequences [22]. Since multiple sRNAs interact with their target mRNAs via a single regulatory core, we reasoned that the conserved 71–77 region of RgsA may be involved in its repression of rpoS expression. To test this hypothesis, a wild-type RgsA overexpression plasmid and its derivative mutant alleles used in our previous study [25] were introduced into a rgsA deletion strain. Northern blot analysis showed that the levels of distinct RgsA-derived RNAs were similar to that of wild-type rgsA expressed from the plasmid (Fig. 4a). Western blot analysis revealed that the RpoS level decreased upon wild-type RgsA overexpression (Fig. 4a). Compared with the control vector, RgsA with deletion of region 58–64 or 65–71 still retained significant negative regulation (Fig. 4a). As expected, deletion of the 71–77 region of RgsA abrogated regulation of rpoS expression (Fig. 4a). Meanwhile, real-time RT-PCR showed that wild-type RgsA and RgsAΔ5–71 reduced rpoS mRNA levels (Fig. 4b). Deletion of the 58–64 region of RgsA only modestly reduced regulation (Fig. 4b). In accordance with the above results, deletion of the 71–77 region restored the rpoS mRNA level (Fig. 4b).

Moreover, the overexpression plasmids of these RNA alleles were transformed into the ΔrgsA::kan mutant carrying the P_{LacO}–rpoS-lacZ fusion vector in order to test their ability to regulate rpoS expression at the post-transcriptional level. As seen in Fig. 4(c), deletion of the 65–71 and 58–64 regions of RgsA had no influence on the regulatory effect on the rpoS-lacZ fusion, whereas RgsAΔ65–77 poorly repressed expression of the rpoS-lacZ fusion. Taken together, these results demonstrate that the 71–77 conserved region of RgsA is necessary for full repression of rpoS expression.

Effect of the rpoS mRNA 5′ UTR on RgsA-mediated rpoS repression

In E. coli and Salmonella, the 5′-leader of the rpoS mRNA has a secondary structure formed by a translation-inhibitory hairpin [16, 32]. To address the question of whether the rpoS leader has an effect on its translation in P. aeruginosa, and to demonstrate the necessary region of rpoS mRNA for regulation mediated by RgsA, we constructed a serial of rpoS-lacZ fusions truncated in the 5′ part of rpoS (Fig. 5a). The regulatory effects on the rpoS2 and rpoS3 truncated fusions mediated by RgsA did not significantly change compared with that of rpoS1 (Fig. 5b). In addition, 5′ truncation up to 25 nucleotides from the AUG start codon supported rpoS-lacZ expression, and RgsA-mediated negative regulation was unaffected (Fig. 5b). Overall, these results confirm that the rpoS leader and the 5′ CDS (–25 through +27) are responsible for translational activity and RgsA-mediated rpoS repression.

DISCUSSION

In this study, we constructed an sRNA library to screen the post-transcriptional regulator of rpoS in P. aeruginosa, and a novel regulator, RgsA, was revealed. The transcription of RgsA was activated by RpoS over the different growth phases, whereas rpoS expression was repressed by RgsA at the exponential phase.

The system developed in this study is a useful tool for determining sRNA regulators of genes of interest in P. aeruginosa. This system is similar to that established for Salmonella in which sRNAs and target translational fusions with GFP are co-expressed from plasmids [33]. In E. coli, the use of an sRNA library was also used to identify sRNAs that regulate a number of genes, e.g. rpoS [34], flhDC [35],...
phoP [36], lrp and soxS [37]. In addition to screen the post-transcriptional regulation of genes of interest, the sRNA overexpression library may also be applied in identifying sRNAs involved in the regulation of pyocyanin synthesis, QS and other virulence factors.

Using our system, we demonstrate that RgsA represses rpoS expression at the post-transcriptional level during the exponential growth phase (Fig. 2). Previous research has revealed that the 71–77 conserved region of RgsA is the regulatory core for interaction with fis and acpP mRNAs, and is critical for its regulatory functions on pyocyanin synthesis and growth rate [25]. Notably, this 71–77 region is also required for the control of rpoS (Fig. 4). Since RgsA is a conserved sRNA in Pseudomonas, our findings indicate that rpoS may be regulated by RgsA in other species of Pseudomonas.

Similar to E. coli and Salmonella, rpoS mRNA in P. aeruginosa has a long UTR consisting of 373 nt, which is involved in translational regulation [16, 29]. Our results suggest that truncation of the rpoS mRNA 5’ UTR to −25 (rpoS4) sufficiently supports rpoS-lacZ expression and RgsA-mediated negative regulation (Fig. 5b). Based on these observations, the putative RgsA-rpoS interaction sites were predicted by Mfold and RNAhybrid [38, 39]. To experimentally assess the putative base-pairing interaction in vivo, compensatory mutations of nucleotides within the predicted interaction sites were introduced into RgsA overexpression and rpoS-lacZ vector,

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**Fig. 4.** Regulation of RpoS expression by RgsA wild-type and mutant RNAs. (a) Western blot (WB) of RpoS levels upon overexpression of RgsA or its mutant alleles when borne on a multi-copy plasmid under the control of the P\text{lac} promoter in P. aeruginosa Δrnga::kan. Samples were collected at an OD\text{\textsubscript{600}} value of 0.7. Levels of RgsA or its mutant alleles were detected by Northern blot (NB) analysis. RpoA and 5S rRNA were used as loading controls, respectively. (b) Real-time RT-PCR analysis of rpoS mRNA levels of the same strains as in Fig. 4(a). The level of rpoS mRNA in a strain harbouring the control plasmid was set at 1, and the rpoS level in each sample was calculated after normalization to the rpoA level. (c) β-galactosidase assay of P. aeruginosa Δrnga::kan strains carrying the P\text{LlacO-}rpoS-lacZ fusion plasmid, each in combination with the control vector pROp200 or plasmids expressing wild-type RgsA RNA or one of the three mutant alleles. Cells were grown to an OD\text{\textsubscript{600}} of 0.7. Error bars represent the sd. Statistical differences * (P<0.05) and ** (P<0.005) between each group were obtained using Student’s t-test.

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**Fig. 5.** Effect of the rpoS 5’ UTR on RgsA-mediated rpoS repression. (a) Schematic drawing of post-transcriptional rpoS-lacZ fusions. The rpoS nucleotides (+1 is A of AUG) fused to lacZ are shown below the bar, and the name of each fusion is shown to the right of the bar. (b) Specific β-galactosidase activities from cells with each P\text{LlacO-}rpoS-lacZ post-transcriptional fusion, in the presence or absence of RgsA overexpressed from plasmid, were assessed when cells were grown to an OD\text{\textsubscript{600}} of 0.7. Error bars represent the sd. Statistical differences ** (P<0.005) between each group were obtained using Student’s t-test.
respective. Then the β-galactosidase assays were performed in *P. aeruginosa* ΔrgsA::Kan. However, the compensatory mutation analysis did not support the predicted base-pairing interaction (data not shown).

Previous studies have shown that OxyS represses *rpoS* translation under oxidative stress, and affects RpoS stability in minimal medium in *E. coli* [17]. However, the regulatory mechanism is still unclear. Although it has been proved that OxyS directly binds to Hfq and *rpoS* mRNA, Hfq has little effect on OxyS binding to *rpoS* [40]. And *rpoS* binds to OxyS much more weakly than to DsrA or RprA [17, 40, 41].

Unlike OxyS, RgsA negatively regulates *rpoS* translation during the exponential growth phase and affects *rpoS* expression at the mRNA level. The nucleases involved in the RgsA-mediated *rpoS* mRNA decrease the need to be further identified. It has been reported that RgsA directly binds to Hfq [26, 31], and our results also revealed that reduction of *rpoS* translation by RgsA is mediated through Hfq (Fig. 3). However, the exact regulatory mechanism on *rpoS* mediated by RgsA is still elusive. It is possible that RgsA represses *rpoS* translation by affecting Hfq activity, or by some other manner blocks access of the positively acting post-transcriptional regulators to the *rpoS* leader, which needs additional experimental evidence.

RpoS has been shown to directly activate RgsA transcription over the different growth phases of the cell [22]. In the exponential growth phase in rich medium, the expression level of RpoS is low, and repressed by RgsA, which would prevent induction of many RpoS-dependent genes, whose expression would be unnecessary and costly. Even though the RgsA level peaks at the onset of the stationary phase, the negative regulation mediated by RgsA on *rpoS* is completely abolished, and RpoS levels therefore increase over the growth phases. This abrogated regulatory effect is likely induced by an unknown regulator, which needs to be identified. In summary, our results have provided a new example of understanding the regulatory network between sRNAs and transcriptional regulators in bacteria.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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