Probing the sRNA regulatory landscape of *P. aeruginosa*: post-transcriptional control of determinants of pathogenicity and antibiotic susceptibility

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Summary

During environmental adaptation bacteria use small regulatory RNAs (sRNAs) to repress or activate expression of a large fraction of their proteome. We extended the use of the *in vivo* RNA proximity ligation method toward probing global sRNA interactions with their targets in *Pseudomonas aeruginosa* and verified the method with a known regulon controlled by the PrrF1 sRNA. We also identified two sRNAs (Sr0161 and ErsA) that interact with the mRNA encoding the major porin OprD responsible for the uptake of carbapenem antibiotics. These two sRNAs base pair with the 5′ UTR of oprD leading to increased resistance of the bacteria to meropenem. Additional proximity ligation experiments and enrichment for Sr0161 targets identified the mRNA for the regulator of type III secretion system. Interaction between the exsA mRNA and Sr0161 leads to a block in the synthesis of a component of the T3SS apparatus and an effector. Another sRNA, Sr006, positively regulates, without Hfq, the expression of PagL, an enzyme responsible for deacylation of lipid A, reducing its pro-inflammatory property and resulting in polymyxin resistance. Therefore, an analysis of global sRNA-mRNA interactions can lead to discoveries of novel pathways controlling gene expression that are likely integrated into larger regulatory networks.

Introduction

A group of bacterial non-coding small RNAs (sRNAs) represent an important arm of global regulatory networks coordinating the expression of multiple genes to optimize the fitness of a microorganism in a particular niche or in response to environmental stressors. These RNA regulators are grouped based on the location of their targets. The *cis*-encoded group includes riboswitches each physically linked to the 5′ region of a single target through base paired structures controlling translation or the stability of the mRNA portion upon an interaction with a ligand (Winkler and Breaker, 2005). Another class of *cis*-encoded regulatory RNAs is transcribed from a strand opposite to the RNA, allowing base pairing through complementarity of the two partially overlapping RNAs. The best studied examples of this group are found among molecular systems controlling plasmid segregation during bacterial cell division, where the expression of a toxin protein is translated only in the absence of the antitoxin sRNA; the antitoxin transcribed from the strand opposite to and partially overlapping the toxin gene disrupts translation and stability of the toxin mRNA by base pairing near the sites of the initiation of translation (Gerdes et al., 1997). The largest group of sRNA regulators are the so-called *trans*-encoded sRNA; these are transcribed usually from intergenic regions and can have multiple targets. The base pairing region of the *trans*-encoded sRNAs share limited complementarity with their target and may involve different regions of the molecules. The base-pairing segments of the majority of mRNAs targeted by sRNA are located in the 5′ untranslated region (5′ UTR), and the sRNA-mRNA interaction frequently occludes the ribosome binding sites (Gottesman and Storz, 2011; Storz et al., 2011). The base pairing, facilitated by the interaction with the RNA chaperone Hfq, blocks translation and recruits ribonuclease, leading to repression of
gene expression. Several exceptions of positive regulation by the sRNAs have been also described, where sRNA base pairing leads to release of the sites of initiation of translation by formation of alternative base paired structures with the 5’ end of the mRNA (Fröhlich and Vogel, 2009; Papenfort and Vanderpool, 2015).

Recently, several investigators have addressed the difficulty of identifying targets of sRNAs using computational tools by developing several experimental approaches towards this goal (Lalaouna and Massé, 2015; Han et al., 2016; Melamed et al., 2016; Waters et al., 2017). Our laboratory has developed and used the method referred to as Global sRNA target Identification by Ligation and Sequencing (GRIL-seq) to identify sRNA interactions with a variety of transcripts, including mRNAs or sequestering RNAs (‘sponges’) (Han et al., 2016). The critical steps used by GRIL-seq include induced expression of a single sRNA in bacteria which is also expressing T4 RNA ligase and subsequent enrichment for transcripts containing the sRNA or, chimeras created by ligation of these sRNAs to base-paired RNAs. The isolated RNA is subjected to RNA sequencing and mapping of the ligated transcripts to the bacterial genome. This allows identification of targets of a specific sRNA. We used GRIL-seq to identify targets of an iron regulated sRNA (PrrF1) (Wilderman et al., 2004) and discovered several unusual regulatory mechanisms, including interaction with a ‘sponge’ derived from the 3’ end of the katA transcript, thus antagonizing the regulatory interaction of the 5’ end of the same mRNA.

To obtain a more global picture of the interactions between the various sRNAs and their targets, we modified the original GRIL-seq method omitting the enrichment step and to facilitate sequencing coverage, depleting the majority of rRNAs from the samples. A computational pipeline was created which allowed us to map the chimeras created by ligation of the 3’ hydroxyl ends of mRNAs to 5’ monophosphate ends of sRNAs and vice versa, thus providing a more comprehensive view of interactions between the individual RNAs in the cell. We refer to the experimental method and the linked data analysis High-throughput GRIL-seq (Hi-GRIL-seq). We validated this approach by first demonstrating that it is capable of detecting the same interactions as GRIL-seq where the samples are enriched for specific sRNA-containing chimeras. Moreover, examination of individual target mRNAs can be traced back to sRNA regulation and we provide two examples of how Hi-GRIL-seq can yield new insights into the interplay between sRNAs and mRNAs within post-transcriptional regulatory networks involving control of expression of important virulence factors.

Results

An assessment of the global sRNA regulatory landscape

To investigate the regulatory range of individual sRNAs, we developed GRIL-seq, an in vivo approach to capture interactions between a single sRNA and its multiple targets, based on preferential ligation of the base-paired transcripts following ectopic expression of T4 RNA ligase (Han et al., 2016). The regulated expression of an sRNA gene allowed us to probe interactions with different transcripts that would vary in abundance when the bacteria are propagated under different growth conditions. We wished to extend this method to a more global approach testing whether endogenously expressed sRNAs can be ligated to their targets without sRNA overexpression or subsequent enrichment for a single sRNA species. Given the performance of current deep sequencing platforms, it should be possible to identify chimeras created by ligation of any sRNA bound to its target by simple sequencing of the entire RNA pool, depleted of the abundant rRNAs. We tested this extension of the GRIL-seq method, referred to as Hi-GRIL-seq, and here we demonstrate its utility as a useful tool for the discovery of global RNA interactions under the steady-state growth conditions. The principles of Hi-GRIL-seq including the data analysis pipeline are outlined in Fig. 1. The major steps include culturing of the bacteria expressing the T4 RNA ligase in the test conditions, RNA isolation, rRNA removal, construction of a library for RNA-seq, paired-end sequencing, and BLAST (Altschul et al., 1990) -based analysis to map the location and abundance of chimeric reads.

To compare Hi-GRIL-seq and GRIL-seq we tracked the interactions of the iron-regulated sRNA PrrF1, a well-characterized regulator of iron homeostasis in P. aeruginosa. This sRNA was used in the development and validation of the original GRIL-seq method (Han et al., 2016). In the present study, instead of expressing PrrF1 under the control of an artificially (L-arabinose) inducible promoter in cells co-expressing the T4 RNA ligase, we relied on induction of expression of this sRNA by shifting cells from iron replete to iron limiting conditions and comparing the identity of the chimeras formed between PrrF1 and its targets.

To confirm that under conditions of iron depletion, PrrF1 is induced and ligated to its regulatory targets, we first identified ligation products formed by PrrF1 and its two known target transcripts (sodB and rubA1) following PCR with reverse transcription (RT-PCR), using sRNA and target gene-specific primer pairs (Fig. 2A). We confirmed the ligation junctions by sequencing of individual amplicons. The presence of unique amplicons was confirmed the ligation junctions by sequencing of individual amplicons.
dependent on induction of the T4 RNA ligase gene \textit{t}4\textit{rl}1 by isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG). The strength of this chimera signal was dose-dependent on iron depletion and thereby \textit{Pr}r\textit{F}1 induction by different concentrations of the iron chelator 2,2\textsubscript{0}-dipyridyl (2,2\textsubscript{0}-DIP), as shown in Supporting Information Fig. S1. Therefore, T4 RNA ligase is able to ligate endogenously expressed sRNAs with their targets.

We performed Hi-GRIL-seq of ligated RNA, recovered from biological duplicate samples of \textit{P. aeruginosa} grown in media where iron was limiting (created by the addition of 2,2\textsubscript{0}-DIP) compared to RNA isolated from cells grown in iron replete media that has not been treated with 2,2\textsubscript{0}-DIP (control condition). Following removal of the majority of rRNAs, cDNA libraries were constructed. Sequencing of these samples using the Illumina platform yielded 64,539,855 and 65,434,990 reads for the iron depletion samples and 79,896,070 and 75,931,749 reads for the iron replete samples. 85–87% of the reads could be mapped to the annotated \textit{P. aeruginosa} PAO1 genome. For accurate identification, a read was called chimeric only when the precise ligation junction between the sRNA and its target could be identified. In total, 0.26–0.29% of the reads were identified to be chimeric reads. We detected no motifs flanking the ligation junctions and the potential bias of ligase’s preference for certain nucleotides was minimal (Supporting Information Fig. S2). The frequency of chimeras was calculated relative to annotated genes and intergenic regions. The list of interactions, where at least 10 chimeras under one of the conditions were identified, can be found in Supporting Information Table S1.

Figure 2B shows the distribution of transcripts found in the \textit{Pr}r\textit{F}1-containing chimeras mapped onto the genomic location of corresponding genes on the PAO1 chromosome. Out of the top 10 targets of \textit{Pr}r\textit{F}1/2 identified by the GRIL-seq method (Han et al., 2016), 8 of them were also identified as the top 15 ranked targets by Hi-GRIL-seq experiments in the iron-depletion condition (Supporting Information Table S2). The unique targets identified by each method may reflect the abundance of mRNAs at the time of induction of RNA ligase. For example, iron limitation in the media by the chelator, used in Hi-GRIL-seq to induce \textit{Pr}r\textit{F}1/2 expression may, lead to the expression of additional targets that were poorly or not expressed under conditions of ectopic expression of \textit{Pr}r\textit{F}1 in iron rich media, employed in the GRIL-seq experiments. The identity of the top \textit{Pr}r\textit{F}1/2 targets was similar between the iron depletion condition and the control condition, indicating that low-level expression of this sRNA occurs even when iron is not limiting (Fig. 2B). However, \textit{Pr}r\textit{F}1/2-containing chimeras were markedly enriched when the expression of \textit{Pr}r\textit{Fs} was induced with 2,2\textsubscript{0}-DIP (note differences in Fig. 2B scales). For example, the chimeric read count for the target \textit{s}dh\textit{C} is 50 and 56 for the duplicate iron depletion groups while the number of chimeras dropped to 2 and 5 for the control groups. Therefore, Hi-GRIL-seq is able to capture sRNA-target interactions in sRNA inducing stress conditions or under conditions when the interactions are abundant. However, its target detection power for sRNA of basal expression level is limited by the sequencing depth. Increasing read depth should uncover new interactions, particularly when the sRNA or its targets are not in high abundance. For interactions involving sRNAs that are highly expressed in the tested condition, increasing read depth has less impact (Supporting Information Fig. S3A).

Identification of new interactions by Hi-GRIL-seq

The global sRNA regulon uncovered by Hi-GRIL-seq can be used to identify specific interactions between sRNAs and any transcript of interest. To illustrate this...
point, we examined two *P. aeruginosa* mRNAs encoding an antibiotic resistance and virulence determinant, *oprD* and *pagL*, respectively (Fig. 3). OprD is an outer membrane porin required for the entry of basic amino acids, peptides and carbapenem antibiotics into the periplasm. Loss of OprD leads to resistance to carbapenem in clinical isolates and enhanced fitness in animal infection models (Pirnay *et al.*, 2002; Wolter *et al.*, 2004; Skurnik *et al.*, 2013b). It has been previously suggested that *oprD* is regulated at the post-transcriptional level (Li *et al.*, 2012; Ducret *et al.*, 2016). *PagL* encodes a lipid A 3-O-deacylase. Deacylation of lipid A modifies its ability to induce Toll-like receptor 4 signaling (Miller *et al.*, 2005).

Hi-GRIL-seq and the analysis of the *oprD*-containing chimeras identified transcripts mapping to two genomic regions on the *P. aeruginosa* PAO1 chromosome, detected in both iron depletion and control groups. The first sRNA is transcribed from a region located between *PA0160* and *opdC* (Fig. 4A). It is annotated as a gene, possibly encoding a short peptide. However, functional studies described below using transcripts with mutations suggest that it is a regulatory sRNA and we will refer to it as Sr0161. The sites of initiation and termination of Sr0161 transcription (184,211–184,458 bp on the plus strand) were confirmed by 3′ and 5′ RACE. The average Sr0161-*oprD* chimeric read count identified by Hi-GRIL-seq of the four samples (from RNA isolated from...
duplicate cultures grown under iron replete and iron limiting conditions) was 28.8 ± 3.9. The analysis of the sequences with the oprD-Sr0161 junctions showed that the ligation occurred between the cleavage products of both the sRNA and mRNA. The majority (93%) of the chimeras consisted of oprD at the 5′ end and Sr0161 at the 3′ end, while the rest were ligation products where the sRNA was at the 5′ end (Fig. 4A). The majority of the oprD ligation junctions resided within 300 bp of the 5′ CDS (Fig. 4A). The second sRNA identified by ligation to oprD mRNA is a previously described sRNA ErsA (Ferrara et al., 2015) (Fig. 4B) with an average chimeric read count of 18.8 ± 3.0. Here, 70% of the products were generated by ligase catalyzed joining of the 3′ hydroxyl group of the oprD fragments and the 5′ monophosphate of various ends of ErsA degradation products, while the remaining had the inverse configuration. The majority of the junctions were either in the 5′ UTR or 5′ CDS of the oprD transcript.

ErsA was reported to negatively regulate algC at the post-transcriptional level (Ferrara et al., 2015), however in all Hi-GRIL-seq samples, the count of algC-ErsA chimeric reads were below 5. This may reflect the low abundance of ErsA and algC in P. aeruginosa PAO1 grown under the experimental conditions used in this study. We therefore examined the interaction between these two RNAs under conditions where ErsA and algC are more abundant, for example, when P. aeruginosa are exposed to D-cycloserine, which induces envelope stress leading to increased alginate production (Lizewski et al., 2002; Wood and Ohman, 2009; Ferrara et al., 2015). D-cycloserine treatment of P. aeruginosa with expression of the T4 RNA ligase, resulted in readily identifiable formation of ErsA-algC chimeras (Supporting Information Fig. S4).

A previously annotated sRNA Sr006 (Wurtzel et al., 2012) transcribed from the intergenic region between 182,570 and 182,693 bp on the minus strand was found in the chimeras with the pagL mRNA (average chimeric read count of the four experiments is 201.3 ± 36.2). The 5′ and 3′ ends of the Sr006 were confirmed by RACE mapping. Unlike the chimeras involving oprD, those between the Sr006 and pagL mRNA were formed exclusively by ligation of 5′ phosphates of pagL fragments to 3′ hydroxyls derived from fragments of sRNA Sr006 (Fig. 4C). The ligation junctions mapped to the 5′ UTR of pagL (Fig. 4C).

In order to confirm the ligations, RT-PCR with primers from each of the two interacting RNAs was performed. First, we determined the kinetics of expression of each sRNA in two P. aeruginosa strains (PAO1 and PA14). The expression of ErsA was increased in stationary
phase of growth compared to exponential phase while no phase dependent expression was observed for Sr0161 and Sr006 (Supporting Information Fig. S5). We then sampled RNA from late exponential phase of growth and identified PCR products using sRNA and mRNA specific primers only in cells where the T4 RNA ligase was induced. Sequencing of these amplicons confirmed that they indeed consisted of sRNA/mRNA chimeras (Supporting Information Fig. S6).

**Negative post-transcriptional regulation of oprD by sRNA**

Base-pairing of sRNAs with mRNAs usually facilitates either degradation or stabilization of the transcripts and GRIL-seq does not differentiate between the potential positive or negative consequences of their interaction.

First, we tested whether deletion or overexpression of the sRNA genes affects the translation of oprD mRNA. We created a recombinant construct, containing (5' to 3') P_tac promoter followed by the predicted 5' UTR and the coding sequence for OprD, ending with additional codons for the FLAG-tag epitope. This IPTG-inducible tagged protein construct was integrated into the chromosome of a *P. aeruginosa* lacking the sRNA genes and carrying either the sRNA overexpression plasmid or control vector. This construct allowed us to test whether the OprD protein levels were affected by posttranscriptional regulation exerted by the sRNAs. Western blot analysis showed that induction of Sr0161 and ErsA caused reductions in OprD protein expression (Fig. 5A).
We further tested the effects of the sRNAs on steady state levels of oprD mRNA levels. Total RNA was extracted from P. aeruginosa Δsr0161 or ΔersA and levels of oprD mRNAs were determined by RT-qPCR (Fig. 5B). The levels of oprD mRNA were quantified relative to those of the housekeeping gene proC (Savili et al., 2003). There was a twofold increase of oprD mRNA comparing Δsr0161 to the parent strain while the deletion of ersA did not cause a significant change in oprD level. However, overexpression of either Sr0161 or ErsA significantly decreased the level of oprD mRNA. Collectively these results show that Sr0161 and ErsA
are sRNA negative regulators of oprD expression, affecting its translation and stability.

The OprD porin of P. aeruginosa is the major channel for entry of the carbapenem antibiotics into the periplasm. Therefore, sRNA-dependent oprD repression should lead to increased resistance against this class of antibiotics. We therefore tested the meropenem susceptibility of P. aeruginosa Δsr0161 and ΔersA and the same strains overproducing the sRNA at two concentrations of the antibiotic and compared the effect to a strain with deleted oprD gene (Fig. 5C). Loss of Sr0161 resulted in a modest decrease in meropenem resistance while strains lacking ErsA were significantly more susceptible to the antibiotic than the wild type PAO1. Overexpression of Sr0161 led to increased resistance, while overexpression of ErsA resulted in only a modest enhancement in survival at the lower antibiotic concentration (0.25 mg l⁻¹) (Fig. 5C, left). Unexpectedly, the CFU of ΔersA strain overexpressing ErsA could not be detected at the higher antibiotic concentrations (0.5 mg l⁻¹) (Fig. 5C, right). It is conceivable that pleotropic effects of ErsA on other targets, affecting meropenem susceptibility, also contributed to this modest effect besides negative regulation of OprD protein expression.

Base pairing interactions between the oprD mRNA and its sRNA regulators

We have generated a model for the most stable favorable pairing between the oprD and Sr0161/ErsA transcripts (Fig. 6A and C). Both sRNAs appear to interact with a partially overlapping sequence of oprD leading to, in each case, the formation of a base paired region which includes the ribosome binding site (the Shine-Dalgarno sequence, S/D) but not the AUG initiation codon. To validate the predicted base-pairing region between Sr0161, ErsA and their target mRNA, we used the β-galactosidase (lacZ) translational fusion to oprD to

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assess the effects of mutations in the predicted base pairing regions. A single nucleotide substitution (G for C) in the middle of the predicted base-pairing segment of Sr0161 abolished the repression of the translational oprD-lacZ reporter. A compensatory mutation (C for G) in the 5' UTR restored the repression of lacZ expression (Fig. 6A and B). Similarly, disruption of the base paired region between ErsA and oprD by two substitutions (CC to GG) abolished repression (Fig. 6C and D). A compensatory mutation in the 5' UTR of oprD could not be constructed because of its effect on translation initiation due to the disruption of the Shine-Dalgarno sequence.

Identification of additional targets of Sr0161 by GRIL-seq

Since we obtained low counts of Sr0161 chimeric reads by Hi-GRIL-seq, other targets could be missed due to the limitation of sequencing depth. Therefore, we used the original GRIL-seq protocol to further extend the repertoire of the Sr0161 mRNA targets, since this approach involves ligation of an expressed sRNA to its targets and an additional step to enrich the transcripts for the sRNAs containing chimeras (Han et al., 2016). Total RNA was isolated from the Δsr0161 strain co-expressing T4 RNA ligase and Sr0161. Following enrichment using complementary oligonucleotides (Supporting Information Fig. S7), the samples were sequenced using the Illumina platform. We obtained 143,383 and 130,811 chimeric reads for the two samples respectively, accounting for 0.51% and 0.54% of the total reads. Among them, 22,270 and 20,504 were reads of chimeric fragments containing sequences from oprD mRNA and Sr0161. The genomic coverage of the top 10 candidate targets are shown in Fig. 7A and Supporting Information Fig. S8. For all of these targets, most of the obtained chimeric reads showed Sr0161 at the 3' end of the fragment. We designed primers against the 7 mRNA targets, and used RT-qPCR to confirm that they are present as chimeras in bacteria expressing T4 RNA ligase (Supporting Information Fig. S8). The RT-qPCR data shows that besides oprD, the RNA levels of four other targets (opdP, braC, lecB, exsA) are also decreased following Sr0161 overexpression and modestly increased by Sr0161 deletion.
The most dramatic effect of Sr0161 overexpression has been seen on the $opdP$ transcript. It has been previously reported that OpdP (PA4501 or OccD3) also contributes to carbapenem uptake and, loss of OpdP causes an increase in carbapenem resistance in strains also lacking OprD (Isabella et al., 2015). However, we were unable to detect a difference in meropenem resistance caused by Sr0161-dependent inhibition of $opdP$ (Supporting Information Fig. S9), suggesting that although it is a target of Sr0161, reduced expression of OpdP by induction of Sr0161 may affect other porin related functions but not show a noticeable change of carbapenem resistance.

Another target was $exsA$, the key transcriptional regulator of the type III secretion system (T3SS). ExsA is an activator protein and controls the expression of the T3SS by specific interactions with regulatory sequences located upstream of the genes coding for the secretion apparatus and the secreted effectors (Yahr and Wolfgang, 2006). We therefore examined whether the Sr0161-dependent repression of ExsA affected the expression of its regulon. Western blot analysis of P. aeruginosa overexpressing Sr0161 in low calcium T3SS inducing medium demonstrated that this sRNA reduced the levels of the PscC outer membrane component of the T3SS machinery as well as that of the secreted exoenzyme S (ExoS), as shown in Fig. 8.

**Positive post-transcriptional regulation of pagL by sRNA**

Another target of an sRNA involved in P. aeruginosa virulence and host adaptation identified by Hi-GRIL-seq is pagL (Fig. 3 and 4C), which was ligated to the sRNA Sr006 (average count 201.3 ± 36.2). To test whether the interaction between Sr006 and the mRNA of pagL had any regulatory effect, we performed western immunoblots on cells expressing PagL with a C-terminal FLAG tag in the wild type PAO1 and D$sr006$ strains overexpressing Sr006 or carrying the empty plasmid (Vec.). The sRNA was expressed for 1 h following the addition of the l-arabinose inducer and expression of the FLAG-tagged PagL was induced with IPTG for 1 h. Samples were collected for western immunoblot analysis. RpoA was used as loading control.

**Fig. 8.** Sr0161 is a regulator of the T3SS. Cultures of P. aeruginosa were grown in T3SS inducing (low Ca$^{2+}$) and non-inducing (high Ca$^{2+}$) media containing the l-arabinose inducer of sRNA expression (Sr0161$^{++}$). Normalized cell pellets and their supernatant were used in western immunoblot and they were probed with antibodies against PscC and ExoS. A blot using antibodies against RpoA served as a loading control.

**Fig. 9.** sRNA Sr006 is a positive regulator of pagL.

A. Western immunoblot analysis of Sr006’s effect on the expression of FLAG-tagged PagL fusion protein. A pTaq directed construct, consisting of a fusion between the 5’ UTR and the entire coding sequence of pagL with the codons of the FLAG tag peptide was inserted into the chromosome of wild type P. aeruginosa PAO1 or the Δsr006 mutant; these strains were then the recipients of the empty vector (Vec.) or Sr006 overexpression plasmid (Sr006$^{++}$). The sRNA was expressed for 1 h following the addition of the l-arabinose inducer and expression of the FLAG-tagged PagL was induced with IPTG for 1 h. Samples were collected for western immunoblot analysis. RpoA was used as loading control.

B. Impact of Sr006 on pagL transcript levels. RT-qPCR was used to assess the pagL mRNA levels in wild type PAO1 or Δsr006. Cells were grown to mid-logarithmic phase and Sr006 expression was induced for 1 h before samples were collected for RNA isolation and RT-qPCR. Housekeeping gene proC was used to calculate relative expression and rpoD was used as a control. Data are shown as mean ± SD for three biological replicates. Statistical comparisons were performed using Student’s t-test. Differences were considered statistically significant at $P$-values < 0.05, and ** denotes $P < 0.01$. 

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enhancement of pagL expression, we mutated two nucleotides (CC to GG) in the sRNA and created compensatory substitutions (GG to CC) in the base-paired region of the pagL 5′ UTR (Fig. 10A). We used a pagL-lacZ translational reporter construct to assess the consequences of these mutations on the expression of the reporter gene. β-galactosidase assays showed that the two-nucleotide substitution mutation in the predicted base-pairing area of Sr006 disrupted its positive regulatory effect on pagL-lacZ translational fusion expression (Fig. 10B). Expression of the mutant Sr006 increased the β-galactosidase activity of translational fusion carrying a compensatory mutation in the 5′ UTR of pagL. We did not identify additional mRNA targets of Sr006 in the tested condition even after carrying out a specific GRIIL-seq experiment with Sr006 overexpression and an enrichment for Sr006 chimeras (data not shown). Therefore, Sr006 appears to control only pagL, although we cannot exclude the possibility that other growth conditions may uncover additional regulatory targets.

To investigate whether regulation of PagL expression by Sr006 can lead to structural changes in the lipid component of LPS, we isolated lipid A from wild type P. aeruginosa strain carrying an empty vector, an sr006 deletion strain carrying an empty vector, and the deletion strain over-expressing Sr006. All strains were grown in M9 succinate minimal media supplemented with 8 μM MgCl₂, a growth condition that activates the two-component regulatory system PhoP/PhoQ. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) in the negative ion mode was used to analyze the structural characteristics of the isolated lipid A. Deacylation of the 3-hydroxy decanoic acid (3-OH C10) acyl chain from lipid A by the PagL enzyme can be observed by MALDI-MS (correlates to a Δm/z of 170). Lipid A structures present in each sample were estimated based on their known structures and molecular weights. The canonical PAO1 lipid A species is penta-acylated with fatty acid chains ranging from 10 to 12 carbons in length (m/z 1446 and 1462). Hexa-acylated lipid A synthesis intermediates differ by the addition of a 10- or 16-carbon acyl chain. These hexa-acylated species can be distinguished using mass spectrometry, allowing identification of the specific PagL substrate (hexa-acylated lipid A with a hydroxy-C10 acyl chain, represented by m/z 1616 and 1632). Compared to PAO1 wildtype (Fig. 11A), the absence of sr006 did not appear to alter the levels of hexa-acylated lipid A species containing C10 (Fig. 11B). However, overexpression of Sr006 reduced the intensity of hexa-acylated lipid A species (Fig. 11C), suggestive of an increase in PagL deacylation. The decrease in 3-OH C10-modified hexa-acylated lipid A species observed with Sr006 over-expression was validated using gas chromatography with flame ion detection (GC-FID) of triplicate samples (data not shown). These results strongly suggest that post-transcriptional regulation of PagL expression by Sr006 has physiological consequences, leading to conversion of lipid A to a less pro-inflammatory form.

It has been previously reported that in Salmonella enterica, PagL increases the polymyxin B resistance of lipopolysaccharide (LPS) aminoarabinose modification-defective strains (Kawasaki et al., 2007). We wished to test whether Sr006-dependent regulation of PagL affects the polymyxin B resistance in P. aeruginosa as well. We found that in a P. aeruginosa strain lacking the LPS aminoarabinose modification (arm) operon, loss of PagL decreased its resistance to polymyxin B, while overexpression of Sr006 lead to increased resistance (Fig. 12).
This result suggests that Sr006 regulates LPS modification through affecting PagL expression.

We evaluated whether the negative and positive regulation by Sr0161 and Sr006 respectively, was dependent on the RNA chaperone Hfq. First, we used RT-PCR to detect chimeras between the sRNAs and their mRNA targets in wild type *P. aeruginosa* or Δhfq each expressing T4 RNA ligase. We observed no specific amplicons of chimeras formed by Sr0161 and the *oprD* mRNA in Δhfq (Supporting Information Fig. S10A) while the absence of Hfq had no effect on the formation of Sr006-pagL chimeras (Supporting Information Fig. S10B). We further tested mRNA levels of target genes by RT-qPCR in the Δhfq strain overexpressing the sRNA or carrying the empty vector. The negative regulation of *oprD* by Sr0161 was abolished in the *hfq* deletion strain (Supporting Information Fig. S10C), consistent with the role of this RNA chaperone in sRNA-mediated gene regulation. In contrast, deletion of *hfq* had no effect on up-regulation of *pagL* by Sr006 (Supporting Information Fig. S10D). The regulation of *pagL* by Sr006 appears to be independent of Hfq and it may require a different RNA chaperone (Attaiech et al., 2017) or no other factor.

**Sequence conservation of sRNAs and their targets**

Previous studies have suggested that two features characteristic of trans-encoded sRNAs play an important role in regulation of gene expression: the conservation of the base-pairing regions and their accessibility (Peer and Margalit, 2011). We determined the conservation of the sRNAs and the 5′ UTR of the targets by calculating the positional entropy as previously described (Peer and Margalit, 2011) (Supporting Information Fig. S11). The entropy score was calculated based on alignment of similar sequences from available complete and draft genomes (from 2226 strains) of *P. aeruginosa* (Winsor et al., 2016) that were found using BLAST (Altschul et al., 1990). No single nucleotide polymorphisms (SNPs) were found in the base-pairing region of either the sRNAs or the 5′ UTR of the targets. A limited number of SNPs was observed outside the base-pairing region of Sr006 and 5′ UTR of *oprD* while the 5′ UTR of *pagL* was almost completely conserved. These results suggest that, in addition to sequences involved in base pairing, other regions of the transcripts of sRNA and their targets are under selective pressure to maintain sequence conservation, possibly to allow optimal base pairing or serving as recognition sites for accessory factors such as Hfq or ProQ. Sr0161 shows considerable
variability, particularly in the 5’ two thirds of the transcript (Supporting Information Fig. S11A). In the Hi-GRIL-seq data, the majority of the Sr0161 chimeras are formed by the ligation of the 3’ hydroxyl group of the target fragments and the 5’ monophosphate of the Sr0161 degradation products at the +171–175 bp downstream of transcription start site (Fig. 4A). We tested whether this 3’ degradation product of Sr0161 alone was sufficient to repress oprD. The 3’ end of Sr0161 starting from the +171 bp containing the base-pairing region (Sr0161-BP) was cloned and this construct, when overexpressed in P. aeruginosa, was able to decrease the RNA level of oprD (Fig. 5B) with a concomitant increase of the resistance to meropenem (Supporting Information Fig. S9B) similar to the effect of the full length Sr0161. Therefore, the 5’ end of Sr0161 is not required for inhibition of oprD and it is conceivable that it is not under the same selection pressure for conservation as the 3’ end.

The predicted secondary structure of Sr0161 and Sr006 and the 5’ UTR of their respective main targets oprD and pagL by the RNA structure software (Supporting Information Fig. S12A–D) showed that the base-pairing regions are all in less structured and more accessible areas. While a putative element associated with Hfq binding could be identified in Sr0161 and oprD, none were apparent in Sr006 or the 5’ UTR of pagL, confirming that the positive regulatory effect resulting from the interaction between these two RNAs is Hfq independent.

Discussion

The recognition of an important regulatory mechanism in bacteria based on base pairing of non-coding sRNAs with complementary regions of mRNAs made it possible to describe the activities of regulatory networks more completely, consisting of both transcriptional and post-transcriptional components. Here, we extended GRIL-seq, the proximity RNA ligation method for sRNA target identification (Han et al., 2016), toward a more comprehensive analysis of sRNA-target RNA interactions. The Hi-GRIL-seq approach differs from GRIL-seq by being able to identify all chimeras created by the expressed T4 RNA ligase in live bacterial cells, followed by RNA-seq using rRNA depleted samples. Hi-GRIL-seq does not require previous knowledge of the sRNA sequence. By comparing RNA ligation products isolated from cultures grown in iron replete and iron-limiting medium, we confirmed that most of the previously identified targets of the iron-regulated sRNA PrnF1 could be detected by Hi-GRIL-seq. The main limitation of the Hi-GRIL-seq approach is sequencing depth, since in most cases, chimeras between the sRNAs and their targets represent a very small fraction of total sequencing reads generated by RNA-seq. It is reasonable to expect that there will be further improvements in the performance of sequencing platforms to increase the depth of sequencing and consequently, enhance the ability of Hi-GRIL-seq to identify a wider range of sRNA-mRNA interactions. However, as we illustrate here, the coverage available by the use of a current sequencing tool (Illumina NextSeq) is sufficient to identify biologically important novel sRNA-mRNA regulatory interactions. In addition, a more important consideration for using Hi-GRIL-seq as a discovery tool for sRNA mediated regulatory network is to sample RNAs from bacteria with a particular genetic background or grown under conditions when a specific sRNA and its targets are expressed at sufficient levels to provide substrates for the formation of chimeras by T4 RNA ligase.

In this work, we showed how Hi-GRIL-seq can be used to uncover novel interactions of sRNAs with specific mRNAs. We found that the levels of a transcript encoding the OprD porin responsible for the uptake of basic amino acids, peptides and carbapenem antibiotics, are controlled by two trans-encoded sRNAs (Sr0161 and ErsA). We confirmed their post-transcriptional regulatory functions by demonstrating that the concentration of the oprD mRNA, the OprD protein level and the antibiotic (meropenem) susceptibility phenotypes are all reduced when the expression of each sRNA is increased. The previously described regulatory target of ErsA, the transcript for the alginate biosynthetic gene algC, was not identified by Hi-GRIL-seq in the non-mucoid strain PAO1, however, interaction between ErsA and algC can be readily detected in several mucoid, alginate producing strains.

Several previous studies have examined the regulatory mechanisms controlling the expression of OprD and suggesting a role for Hfq and an sRNA-based regulatory mechanism. The transcription of the oprD gene is negatively regulated by two metal responsive two-component systems (CzcRS and CopRS) and this regulatory effect requires the Hfq protein (Ducret et al., 2016). Hfq appears to directly facilitate binding of the response regulator CzcR to the promoter of the oprD gene. The molecular mechanism for the involvement of an RNA chaperone in transcriptional repression has not been elucidated to date. The PhrS sRNA, identified by Hfq co-immunoprecipitation was shown upon overexpression to increase the intracellular concentration of OprD (Sonnewitter et al., 2008). Whether this effect is indirect or the consequence of positive regulation via base-pairing interaction between PhrS and oprD has not been determined. It is therefore conceivable that Hfq and multiple sRNAs, including the two identified in this study, regulate OprD. The transcription of the sRNAs may be under regulatory control of environmental

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signals that reflect the diverse functions of this porin ranging from the uptake of nutrients, antibiotics and a role in recognition by host defenses (Skurnik et al., 2013a,b).

Following enrichment for Sr0161 containing chimeras and sequencing (GRIL-seq), we identified additional targets of this sRNA and for braC, oprD, lecP and exsA, and confirmed its regulatory effects using P. aeruginosa lacking or over-expressing the sRNA (Fig. 7). Of particular interest is the interaction of Sr0161 with the transcript encoding the T3SS regulator ExsA, adding another element to the complex regulatory network controlling this virulence mechanism by various transcriptional and post-transcriptional mechanisms (Yahr and Wolfgang, 2006; Diaz et al., 2011). The role of Hfq in post-transcriptional regulation of the T3SS has been established by demonstrating its role in stabilizing the RsmY sRNA (Sorger-Domenigg et al., 2007). RsmY is an antagonist of the RsmA protein, a positive regulator of the T3SS (Burrowes et al., 2006). Therefore, Hfq appears to indirectly and adversely affect T3SS expression by causing an increase in levels of RsmY. Our finding that Sr0161 directly interacts with the 5’ UTR of the exsA mRNA leading to its degradation and consequently a block in the synthesis of at least one component of the T3SS machinery and a T3SS effector, implicates another sRNA-mediated mechanism for regulating this virulence mechanism during infection.

Sr006 is another sRNA uncovered by the application of Hi-GRIL-seq, regulating the expression of a determinant of pathogenesis. Over-expression of this sRNA causes an increase in the levels of the pagL transcript, encoding a lipid A 3-O-deacylase, which leads to a reduction in the pro-inflammatory signaling activity of LPS (Moskowitz and Ernst, 2010). As expected, over-expression of Sr006 in P. aeruginosa results in increased lipid A decylation (Fig. 11) and a corresponding enhancement of polymyxin B resistance (Fig. 12). PagL levels were shown to be elevated in the isolates from young CF patients compared to strains from other sources and mutations in pagL are not uncommon among isolates from patients with a severe respiratory disease (Ernst et al., 2006; Greipel et al., 2016). A decrease of expression of this sRNA during the chronic phase of the infection could lead to an increase in the highly inflammatory hexa-acylated lipid A in the bacterial LPS, contributing to the severe lung damage seen in CF patients with more advanced disease.

Unlike the negative control of oprD translation or stability by two sRNAs, pagL is positively regulated by Sr006 without a need for Hfq. Regulation of expression of the PagL protein, likely at the level of transcription of Sr006 in response to its interaction with the infected host may represent a mechanism allowing P. aeruginosa to recognize the host environment. An increase in the levels of pagL, in response to contact with epithelial cells has been previously demonstrated (Gellatly et al., 2012). Whether this effect on transcript levels is due to increased transcription of the pagL gene or a stabilizing effect by Sr006 on the mRNA, has not been determined.

We identified several interactions between individual sRNA which may result in regulatory functions. Among these, the CrcZ sRNA, involved in post-transcriptional regulation of catabolic genes (Sonnleitner et al., 2014) interacted with PrrF1 as well as Sr0161 (Figs. 2B and 7A). It is conceivable that CrcZ can recognize both of these sRNAs by a base-pairing mechanism, and sequesters them from their mRNA targets by a ‘sponging’ mechanism. A more likely explanation is that the formation of the chimeras between CrcZ and PrrF1 or Sr0161 by RNA ligase is through its interaction with Hfq (Sonnleitner et al., 2017), where simultaneous binding of the sRNA to this protein may lead to the formation of a covalently linked product due to the proximity of their 3’ and 5’ ends.

In conclusion, the work described here demonstrates that Hi-GRIL-seq, a modification of the T4 RNA ligase catalyzed proximity ligation method, is well suited for studies of sRNA-mediated regulation of gene expression at a genome-wide level. By focusing on a handful of examples, we demonstrated that Hi-GRIL-seq is a promising tool for the discovery of novel sRNA-mediated regulatory pathways. Refining this method, particularly when improved platforms with an increased sequencing depth to detect interactions between less abundant sRNA and their targets become available, should greatly facilitate progress in our attempts to fully define complex regulatory networks involved in bacterial adaptation to different environments including an infected host.

**Experimental procedures**

**Bacterial strains and plasmids**

The bacterial strains and plasmids used in this study are listed in Supporting Information Table S3. Unless otherwise noted, P. aeruginosa PAO1 was used for plasmid conjugation and mutant construction. All deletion and substitution mutants of P. aeruginosa were created with the pEXG2 plasmid as described previously (Rietsch et al., 2005). A list of the oligonucleotides used for the constructions is provided in Supporting Information Table S4. Cloning was performed with either splicing by overlap extension (SOEing) PCR (Horton et al., 1989) and T4 DNA ligation or a Gibson Assembly cloning kit (New England Biolabs). The sRNA overexpression vectors were created by coning PCR-generated amplicons starting from the sRNA transcription start sites to their native transcription terminators, inserted
was added to 300

The cells were then collected for RNA extraction. For GRIL-group while the controls were not treated with the chelator.

et al previously (Han et al., 2016). Briefly, expression of T4 RNA ligase was induced for 1 h with 1mM IPTG at OD600 0.5, T4 RNA ligase was induced by addition of IPTG to 1mM. After 1 h, the iron chelator 2,2'-dipyridyl (2,2'-DIP) was added to 300 μM for 20 min for the iron depletion group while the controls were not treated with the chelator. The cells were then collected for RNA extraction. For GRIL-seq, P. aeruginosa PAO1 carrying pKH13-t4rnl1 was grown overnight in LB broth with carbenicillin at 37°C.

These cultures were then used to inoculate the same medium to an OD600 0.01. When the cultures reached OD600 0.5. T4 RNA ligase was induced by addition of IPTG to 1mM. After total RNA isolation, the ribosome integrity number (RIN) was determined with an RNA picochip on a 2100 Agilent Bioanalyzer. The RINs ranged from 9 to 10. For Hi-GRIL-seq, the Ribo Zero rRNA removal kit for Gram-negative bacteria (Illumina) was used for rRNA depletion. For GRIL-seq, the enrichment of chimeric sRNA was carried out as previously described (Han et al., 2016), with the exception that poly(A)-tailed Sr0161 complementary oligonucleotides were used to capture chimeric sRNA, their sequence is listed in Table 4. After rRNA depletion or sRNA enrichment, cDNA libraries were prepared with the NEB-Nex Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). RNA-seq was performed on an Illumina NextSeq platform by the Biopolymers Facility at Harvard Medical School.

Preparation of the cDNA library for illumina sequencing

A custom BLAST-based chimera pipeline was used to analyze the GRIL-seq data. The first and last 25 nucleotides at both ends of the Illumina reads were mapped to a reference genome using a modified version of the Rockhopper software system (McClure et al., 2013). Reads were considered to be potentially chimeric if the two ends mapped to different regions of the genome at least 1000 nucleotides apart and the mappings contained no more than three mismatches. This allowed rapid identification of a subset of the reads that were likely to be chimeric. These potential chimeric reads were further aligned to the genome using BLAST version 2.6.0. BLASTN was run with the expectation value (e-value) set to 0.01, reward value set to 1, penalty value set to -2 and other parameters set to default (States et al., 1991). The precise nucleotide locations of the chimeric junctions were determined from the BLAST results by the following criteria: (i) the read did not have a long top hit (read length-20, e-value ≤ 1.0e-50) suggesting that the read was likely to correspond to a single fragment rather than a chimeric read; (ii) the read had at least two BLAST hits with e-value ≤ 0.01, and the two hits were either adjacent, or with up to five nucleotides of gap or overlap; (iii) for reads that aligned to multiple chimeric locations in the genome, the most significant alignment as determined by e-value was used and (iv) reads that mapped to different regions of at least 2000 nucleotides apart were used for downstream analysis. We evaluated different distance thresholds (Supporting Information Fig. S14), and found that 2000 is efficient in removing chimeras that are likely to be preferential ligations within the same transcript. After determining the chimeric junctions, we calculated the frequency of junctions corresponding to annotated protein coding genes, RNAs, and intergenic regions, as reported in Supporting Information Table S1. When reporting top candidate targets for an sRNA (Supporting Information Table S2

was performed with 32 cycles instead of 30. The PCR products were separated by 2% agarose gel electrophoresis, and cloned into pJET1.2 vector (Thermo Fisher) and sequenced with the pJET1.2 forward and reverse primer.

Media and growth conditions

P. aeruginosa cultures were grown with shaking at 37°C, in Luria–Bertani (LB) broth. Antibiotics were added to the culture as required at the following concentrations unless otherwise noted: for P. aeruginosa: 150 μg ml⁻¹ carbenicillin, 75 μg ml⁻¹ gentamicin, 75 μg ml⁻¹ tetracycline and 25 μg ml⁻¹ igrasan; for E. coli: 50 μg ml⁻¹ carbenicillin, 15 μg ml⁻¹ gentamicin and 10 ml⁻¹ tetracycline. For lipid A analysis, P. aeruginosa were grown in M9 minimal media supplemented with 20 mM succinate and 8 μM MgSO₄.

For RT-PCR detection of PrfF1 chimeras and for Hi-GRIL-seq, P. aeruginosa PAO1 carrying pKH13-t4rnl1 was grown overnight in LB broth with carbenicillin at 37°C. These cultures were then used to inoculate the same medium to an OD₆₀₀ 0.01. When the cultures reached OD₆₀₀ 0.5. T4 RNA ligase was induced by addition of IPTG to 1mM. After 1 h, the iron chelator 2,2'-dipyridyl (2,2'-DIP) was added to 300 μM for 20 min for the iron depletion group while the controls were not treated with the chelator. The cells were then collected for RNA extraction. For GRIL-seq, PAO1Sr0161 carrying two expression plasmids pKH13-t4rnl1 and pKH6-Sr0161, was grown as described previously (Han et al., 2016). Briefly, expression of T4 RNA ligase was induced for 1 h with 1mM IPTG at OD₆₀₀ 0.5, followed by induction of sRNA Sr0161 for 20 min with 0.2% L-arabinose for 20 min.

RNA isolation and reverse transcription

Bacteria were harvested by centrifugation for 1 min at 16,000 × g when they reached a density equivalent to OD₆₀₀ 1.0 and the pellets were rapidly frozen in liquid nitrogen. Total RNA was isolated using the phenol-free total RNA purification kit (Amresco), according to the manufacturer’s instructions, with the modification that 50 μl of nuclease-free water (Ambion) was applied to the column for RNA elution. A NanoDrop spectrophotometer (Thermo Fisher) was used to measure RNA concentration. To remove DNA from total RNA samples, 10 μg of RNA sample was treated in a 50 μl reaction with a Turbo DNA-free kit (Ambion). To prepare template for RT-PCR and RT-qPCR, cDNA was synthesized with a SuperScript III First-Strand Synthesis system (Invitrogen) with random hexamers.

Detection of sRNA-target chimeras by RT-PCR

cDNA reverse transcribed from 100ng of total RNA was used as template for RT-PCR with a GoTaq Green Master Mix (Promega). Primers specific for sRNA and mRNA respectively were used to amplify chimeras and are listed in Supporting Information Table S4. Cycling conditions were: 95°C for 3 min; 30 cycles of 94°C for 25 s, 55–66°C for 25 s and 72°C for 60 s; and a final extension cycle of 72°C for 5 min. For detection of chimeras formed by Sr0161 and the mRNA of exsA, PA3801 and wbpH, PCR was performed with 32 cycles instead of 30. The PCR products were separated by 2% agarose gel electrophoresis, and cloned into pJET1.2 vector (Thermo Fisher) and sequenced with the pJET1.2 forward and reverse primer.

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for PrfF1/2), we considered these as chimeric reads if the sRNA corresponded to either the 5’ end or the 3’ end of the chimera. Chimeric junctions located in the untranslated region and the coding region of a target gene were combined when calculating the abundance of chimeric reads of a target with a given sRNA. Visualization of chimeric junctions throughout the genome was performed using R and IGV (Thorvaldsdóttir et al., 2013).

Western immunoblots

The plasmid pPtac-miniCTX:FLAG was first constructed for making IPTG inducible c-terminal FLAG tagged fusion protein. Briefly, plasmid miniCTX1 was linearized with KpnI and SacI and a synthesized 1.6 kb fragment (gBlocks gene fragment, IDT), containing the sequence for lacIq, P tac, a multiple cloning site followed by a C-terminal FLAG tag and stop codon, was inserted (Supporting Information Table S4). The lacIq and P tac sequence is the same as in pPtac-miniCTX::lacZTL (Han et al., 2016). The 5’ UTR and ORF without a stop codon of oprD and pagL were inserted into the pPtac-miniCTX::FLAG at EcoRI and BamHI restriction sites. The construct was introduced into the chromosome of various P. aeruginosa strains at the CTX attB site. For Western blot analysis, overnight cultures of P. aeruginosa containing the FLAG tagged fusion protein and the pKH6 empty vector, or the sRNA overexpression plasmid, were grown in LB with gentamicin and tetracycline. The overnight cultures were diluted to OD600 0.02 in the same medium and incubated at 37°C to OD600 0.5. sRNA was induced for 1 h with 0.2% L-arabinose and then FLAG fusion protein was induced with 0.1 mM IPTG for 1 or 3 h, followed by sample collection. Total proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. FLAG tagged fusion proteins were detected with rabbit anti-FLAG antibody (Sigma). The loading control RpoA was detected with mouse anti-E. coli RNA Polymerase α subunit antibody (Yang et al., 2015).

Western immunoblot was performed to detect the protein level of ExoS and PscC in strains expressing high or low levels of Sr0161. P. aeruginosa overnight cultures were diluted to an equivalent of OD600 0.02 in low-calcium or high-calcium LB media. Low-calcium medium was achieved by treating LB media with nitritriacetic acid (NTA) to 10 mM. For high-calcium medium, CaCl2 was added to the NTA-treated LB to a final concentration of 20 mM (Wolfgang et al., 2003). The media was supplemented with 0.2% L-arabinose to induce Sr0161 expression. Cultures were incubated at 37°C for 6 h and reached OD600 2.2–2.4. All samples were normalized based on OD600. For each sample, half of the culture was centrifuged and the cell pellets were resuspended in Laemmli sample buffer (Bio-Rad). The supernatant of the other half of the sample was precipitated with 10% trichloroacetic acid, washed twice with acetone, and resuspended in sample buffer. Western immunoblot was performed similarly as described above except that ExoS and PscC were detected with respective primary polyclonal antibody (Lee et al., 2005; Hoang et al., 2011).

RT-qPCR

For determining target RNA levels upon induction of sRNA, overnight cultures of PAO1 or PAO1 sRNA deletion mutants, carrying either the empty plasmid pKH6 or pKH6-sRNA, were grown in LB with gentamicin. The following morning, the cultures were diluted to an OD600 0.02 and the bacteria were grown at 37°C with shaking. When OD600 reached 0.5, sRNA was overexpressed for 1 h by adding L-arabinose to 0.2%. RNA extraction, DNA removal and cDNA synthesis were performed as described above. Quantitative PCR was carried out with the PerfeCTa SYBR Green FastMix (Quanta Biosciences) on a Mastercycler Realplex2 system (Eppendorf) according to the manufacturer's instructions. The primers used for RT-qPCR are listed in Supporting Information Table S4. RNA level of genes relative to the housekeeping gene proC (Savli et al., 2003) were calculated.

β-galactosidase assay

The lacZ translational fusion was constructed as described previously (Han et al., 2016). Briefly, the sequence consisting of the 5’ UTR and the codons for the first 20 amino acids of oprD or the sequence of the 5’ UTR and the codons for the first 18 amino acids of pagL were amplified by PCR and inserted between the EcoRI and HindIII sites of pPtac-miniCTX::lacZTL. The base-pairing region mutation constructs were created by SOEing PCR with primers containing the changes. The translational lacZ reporter constructs were integrated into the chromosome at the attB site. Overnight cultures of cells containing the lacZ reporter construct and the pKH6 empty vector or sRNA overexpression plasmid were grown in LB with gentamicin and tetracycline. The overnight cultures were diluted to OD600 0.02 in the same medium and incubated for additional 4 h and reached OD600 0.5–1. The sRNA expression was induced for 1 h with 0.2% L-arabinose and then lacZ fusion protein was induced with 0.1 mM IPTG for 2 or 4 h, followed by β-galactosidase assay, as performed in an earlier publication (Griffith and Wolf, 2002).

5’ and 3’ rapid amplification of cDNA ends (RACE)

5’ and 3’ RACE was carried out using the FirstChoice RLM-RACE Kit (ThermoFisher) according to the manufacturer’s instructions with modifications as described in a previous paper (Sharma et al., 2014). For 5’ RACE, the 5’ monophosphate end of Tobacco Acid Pyrophosphatase (TAP) treated RNA was ligated to the 5’ RACE adapter by T4 RNA ligase. Samples without TAP treatment was used as control. Random-primed reverse transcription reaction and nested PCR amplified the 5’ end of the sRNA. For 3’ RACE, the total RNA was polyadenylated using Poly (A) Polymerase enzyme (New England Biolabs). Polyadenylated RNA was then reversed transcribed and PCR amplified for the 3’ end of the sRNA. The PCR products were cloned into pJET1.2 vector (Thermo Fisher) and sequenced to confirm the initiation and termination site of sRNA transcription. Primers used for 5’ and 3’ RACE are listed in Supporting Information Table S4.
**Lipid A structural analysis**

For a mass spectrometric analysis, cell pellets were processed with an ammonium hydroxide-isobutyric acid-based procedure (El Hamidi et al., 2005) to extract the lipid A. Briefly, approximately 50 ml of cell culture was pelleted, resuspended in 400 μl of 70% isobutyric acid and 1M ammonium hydroxide (5:3, vol/vol), and heated for 1 h at 100°C. After incubation, samples were centrifuged at 2000 × g for 15 min and supernatants were transferred to a fresh tube with endotoxin-free water (1:1 vol/vol). Samples were frozen on dry ice and lyophilized overnight. Dried material was washed twice with methanol and lipid A was extracted with 100 μl of a mixture of chloroform, methanol, and water (3:1:0.25, vol/vol/vol). After extraction, 1 μl of the lipid A concentrate was spotted on a stainless steel matrix-assisted laser desorption-time of flight (MALDI-TOF) plate followed by 1 μl of 10 mg/ml norharmane matrix in chloroform:methanol (2:1, vol/vol) (Sigma-Aldrich, St. Louis, MO). Samples were air dried and then analyzed on a Bruker Microflex mass spectrometer (Bruker Daltonics, Billerica, MA) in the negative-ion mode with reflectron mode. Electrospray tuning mix (Agilent, Palo Alto, CA) was used for mass calibration. Spectral data were processed and analyzed with Bruker Daltonics FlexAnalysis software. For gas chromatography analysis, LPS was extracted from biological triplicate replicates of each strain as described previously (Somer ville et al., 1996). Briefly, 80 mg of cell pellet was resuspended in 90% phenol and water (1:1, vol/vol) and incubated for 1 h at 70°C with constant shaking. Samples were cooled and centrifuged at 9000 × g for 10 minutes. The supernatant was transferred to a fresh glass tube and washed twice with diethyl ether. The lower phase containing LPS was collected and lyophilized. Fatty acids of the resultant product were converted to methyl esters by methanolysis (2M HCl in methanol; Alltech, Lexington, KY) with 18 h incubation at 90°C. Samples were extracted twice with hexane. GC analysis was conducted using an HP 5890 series II with a 7873 autoinjector. Fatty acid methyl esters (Matreya BAME/bacterial acid methyl esters, CP mix no. 1114) and pentadecanoic acid (C15; Sigma, St. Louis, MO) were used as standard of a known fatty acid mixture and internal standard for concentration control, respectively.

**Prediction of interactions and secondary structures of sRNAs and target mRNAs**

We used the IntaRNA (Busch et al., 2008) algorithm to predict base pairing between sRNA and target RNAs. RNAstructure (Reuter and Mathews, 2010) software was used to predict secondary structure of the sRNAs and target RNAs.

**Computing positional conservation of sRNA and target sequence**

Similar sequences were identified by BLAST from 2226 complete and draft genomes of *P. aeruginosa* (Winsor et al., 2016). A multiple sequence alignment of the similar sequences was performed using Clustal Omega. A conservation entropy score for each position was calculated as previously described (Peer and Margalit, 2011).

**Data availability**

The sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession codes SAMN07284097-SAMN7284100.

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**Author Contributions**

YFZ, SL, KH, CEC, RKE designed the study, YFZ, CEC performed the experiments, YFZ, KH, SL, RKE and BT analyzed the data and YFZ, SL wrote the manuscript.

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Supporting information

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