A rhlI 5′ UTR-Derived sRNA Regulates RhlR-Dependent Quorum Sensing in Pseudomonas aeruginosa

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ABSTRACT N-Acyl homoserine lactone (AHL) quorum sensing (QS) controls expression of over 200 genes in Pseudomonas aeruginosa. There are two AHL regulatory systems: the LasR-LasI circuit and the RhlR-RhlI system. We mapped transcription termination sites affected by AHL QS in P. aeruginosa, and in doing so we identified AHL-regulated small RNAs (sRNAs). Of interest, we noted that one particular sRNA was located within the rhlI locus. We found that rhlI, which encodes the enzyme that produces the AHL N-butanoyl-homoserine lactone (C4-HSL), is controlled by a 5′ untranslated region (UTR)-derived sRNA we name RhlS. We also identified an antisense RNA encoded opposite the beginning of the rhlI open reading frame, which we name asRhlS. RhlS accumulates as wild-type cells enter stationary phase and is required for the production of normal levels of C4-HSL through activation of rhlI translation. RhlS also directly posttranslationally regulates at least one other unlinked gene, fpvA. The asRhlS appears to be expressed at maximal levels during logarithmic growth, and we suggest RhlS may act antagonistically to the asRhlS to regulate rhlI translation. The rhlI-encoded sRNAs represent a novel aspect of RNA-mediated tuning of P. aeruginosa QS.

IMPORTANCE The opportunistic human pathogen Pseudomonas aeruginosa possesses multiple quorum sensing systems that regulate and coordinate production of virulence factors and adaptation to different environments. Despite extensive research, the regulatory elements that play a role in this complex network are still not fully understood. By using several RNA sequencing techniques, we were able to identify a small regulatory RNA we named RhlS. RhlS increases translation of RhlI, a key enzyme in the quorum sensing pathway, and represses the fpvA mRNA encoding one of the siderophore pyoverdine receptors. Our results highlight a new regulatory layer of P. aeruginosa quorum sensing and contribute to the growing understanding of the role regulatory RNAs play in bacterial physiology.

KEYWORDS term-seq, transcriptome, small RNA, Hfq, pyoverdine

The opportunistic pathogen Pseudomonas aeruginosa, like many bacteria, has the ability to sense its population density and respond to environmental changes by initiating a gene regulatory system termed quorum sensing (QS). In proteobacteria like P. aeruginosa, QS commonly involves diffusible N-acylhomoserine lactone (AHL) signaling molecules that are recognized by corresponding transcription factors. When the population density, and thus signal concentration, reaches a critical threshold, a coordinated, population-wide shift in gene expression occurs. This facilitates P. aeruginosa adaptation to its environment (1, 2). In P. aeruginosa, there are two AHL QS systems: the Las system and the Rhl system. The Las system consists of LasI, which catalyzes synthesis of N-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL), and the

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3OC12-HSL-dependent transcription factor LasR. The Rhl system consists of RhlI, the N-butanoyl-homoserine lactone (C4-HSL) synthase, and the C4-HSL-dependent transcription factor RhlR. Together these two QS circuits activate over 200 genes (3–7). Although much is known about direct control of *P. aeruginosa* genes by LasR and RhlR, less is known about QS-mediated posttranscriptional regulation by noncoding RNA elements.

Over the last two decades, we have learned that noncoding RNA is critical for the posttranscriptional control of gene expression. Posttranscriptional regulation by small regulatory RNAs (sRNAs) is known to occur by two mechanisms: by direct base pairing to target mRNAs or by binding to proteins. In the class of base-pairing sRNAs, trans-encoded sRNAs base pair with limited complementarity to target mRNAs mediated by the RNA chaperone protein Hfq and recently discovered ProQ domain-containing proteins (reviewed in reference 8). Antisense RNAs (asRNAs), the second type of base-pairing sRNA, are encoded on the strand opposite to that encoding the mRNA and base pair with the mRNA, generally in the absence of protein chaperones, to regulate either translation or stability of their target mRNA (reviewed in references 9 and 10).

RNA-mediated control of transcription or translation can also involve riboswitches or thermosensors found within the 5′ untranslated regions (UTRs) of some genes or operons. They control gene expression or translation by directly binding metabolites or signaling molecules, by sensing pH, or by sensing changes in temperature (11, 12). In the absence of a canonical riboswitch, the secondary structure of the RNA in the 5′ UTR can on occasion regulate translation by sequestering access to a ribosome-binding site (RBS). For example, in *P. aeruginosa* the sRNA PhrS regulates expression of *pqxR*, which codes for a transcriptional activator, by binding the RBS of an upstream open reading frame (uORF). The binding alters the RNA structure to activate translation of the uORF, which by translational coupling leads to *pqxR* translation (13). Recent studies suggest riboswitches, 5′ UTRs, sRNAs, and asRNAs can regulate each other, thus forming complex regulatory networks (14, 15).

Given the complexity of RNA-based regulation, it is not surprising that sRNAs and QS are intricately linked. For example, similar to other bacteria, *P. aeruginosa* Hfq mutants grow abnormally and are attenuated for virulence (16). In the context of QS, transcript profiling showed that Hfq influences expression of 72 QS-activated genes (16–18). More recently, a high-resolution transcriptome sequencing (RNA-seq) study identified a number of sRNAs, including two that were induced by the LasR QS system (19), while another study identified the sRNA PhrD as a positive regulator of *rhlR* (20). Independent of sRNA-based regulation, two RNA thermometers were shown to control expression of the RhlR-activated *rhlAB* operon, as well as *lasI* (21). Although *P. aeruginosa* genome-wide RNA-seq studies have identified hundreds of potential sRNAs and asRNAs (18, 19, 22–25, 28), none have focused specifically on identifying regulatory RNA elements controlled by QS.

We have used term-seq (26) to quantitatively map 3′ ends of RNA in *P. aeruginosa* and identify those ends affected by QS. We did not identify any QS-responsive riboswitches, but we did identify a number of sRNAs not previously associated with QS. There was a strongly QS-induced transcription termination site in the 5′ UTR of *rhlI*. Follow-up investigations led us to describe an sRNA we name RhlS, which is derived from the 5′ UTR of *rhlI*. RhlS activates translation of *rhlI* and induces C4-HSL production, which can be partially complemented in trans. RhlS also acts posttranscriptionally in trans to regulate the *fpvA* mRNA, which encodes a siderophore receptor (27). Furthermore, the term-seq analysis revealed an antisense RNA opposite *rhlI*. We call this antisense RNA asRhlS and present evidence that RhlS may act as an asRhlS antagonist in *P. aeruginosa*.

**RESULTS**

**Identification of QS-induced RNA 3′ ends by term-seq analysis.** We mapped the RNA 3′ termini, representing to a large extent the transcription termination sites (TTSs) in a *P. aeruginosa* PAO1 LasI, RhlI (AHL synthesis) mutant incubated with or without

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added 3OC12-HSL and C4-HSL by using term-seq as diagrammed in Fig. 1A and described in detail in the supplemental material (see Text S1). We identified a total of 804 TTSs associated with annotated 

\[ \text{P. aeruginosa} \] 

genes or operons (see Table S1, tab A, in the supplemental material).

In addition, we identified 21 RNA termini whose expression levels were elevated or reduced by AHLs (Table 1; Table S1, tab B). We believe this is an underrepresentation of QS-regulated genes because of our stringent analysis criteria and limited experimental conditions. Most of the sites affected by AHLs correspond to the 3' ends of known QS-regulated genes. As an example, term-seq reads depicting the 3' end of \[ \text{lasB} \], which codes for the QS-induced elastase enzyme, were much more abundant in cells incubated with AHLs than in cells without AHLs (Fig. 1B; Table S1, tab B). We also identified AHL-controlled TTSs for a number of previously identified sRNAs not known to be associated with QS (Table 1; Table S1, tab B). Among those regulated by AHLs, two previously identified sRNAs (28) are shown in Fig. 1C and D. Interestingly, one of these sRNAs, designated SPA0034 (28), is located downstream of a known QS-activated gene, \[ \text{PA1869} \] (5), and might be a 3' UTR-derived sRNA similar to those identified in other bacterial species (29–32). Notably, we identified a premature tran-

### TABLE 1 Known small RNAs identified by term-seq as differentially regulated by AHLs

| sRNA name | Flanking genes (5'/3') | 3' end positiona | sRNA strand | Fold changeb | Comments and reference(s) |
|-----------|------------------------|------------------|-------------|--------------|--------------------------|
| RhlS/SPA104 | rhlR/rhlI           | 3889777         | -         | 55.68        | Fig. 2A (28)            |
| SPA0116   | PA2768/PA2769         | 3127925         | +         | 28.36        | PA2769, known to be QS regulated (28, 54) |
| SPA0079   | PA2763/PA2764         | 3123367         | -         | 18.05        | Fig. 1C (28)            |
| SPA0034   | PA1869/PA1870         | 2031856         | +         | 3.06         | Fig. 1D (28)            |
| SPA0080   | PA2789/PA2790         | 3147657         | +         | 2.94         | 28                       |
| AmiL      | amiE/PA3367           | 3778033         | -         | 2.92         | 18                       |
| pant90    | PA0806/PA0807         | 884182          | -         | 2.26         | 23                       |

aIndicates position of the 3' end signal in the PAO1 genome.

bIndicates fold change with or without AHLs determined as differentially expressed if they changed by more than 2-fold with a \( P \) value of <0.05.
Additionally, there is a TTS upstream of the rhlR gene in AHL-induced cells was over 50 times higher than in uninduced cells. The AHL-induced RNA 3' end in the rhlI UTR is 34 nucleotides upstream of the rhlI start codon, and expression of this RNA terminates in AHL-induced cells was over 50 times higher than in uninduced cells (Fig. 2A). Additionally, there is a TTS upstream of the rhlR gene in AHL-induced cells was over 50 times higher than in uninduced cells. The gap in RNA-seq coverage within rhlI is due to the deletion of the ORF. The number of reads at the term-seq site (black pillar) was determined as in Fig. 1 (Text S1). The RNA-seq coverage shown is not strand-specific. (B) Northern analysis of RhlS expression in PAO1 ΔlasI ΔrhlI (MPK0493). Overnight cultures of PAO1 ΔlasI ΔrhlI were grown as described (Text S1). At an optical density (OD600) of ~0.8, the cultures were split and no AHL or both C4-HSL (10 μM) and 3OC12-HSL (2 μM) were added to the cultures. Cells were harvested after 10 and 60 min, RNA was extracted and 10 μg total RNA was analyzed by Northern blotting with a 32P-labeled oligonucleotide specific to RhlS or 5S as a loading control. RNA from the ΔrhlS-l (MPK0627) strain collected at OD600 of 2.0 was used as a control for band specificity.

**Transcription termination in the 5' UTR of rhlI.** The AHL-induced RNA 3'-end in the rhlI UTR is 34 nucleotides upstream of the rhlI start codon, and expression of this RNA terminates in AHL-induced cells was over 50 times higher than in uninduced cells (Fig. 2A). Additionally, there is a TTS upstream of the rhlI gene that maps to the end of the rhlR ORF. Thus, the 3' end detected in the 5' UTR of rhlI is not likely due to transcriptional read-through from rhlR. We analyzed whole transcriptome RNA-seq data and found that AHLs induced expression upstream of the rhlI UTR termination signal by about 100-fold over uninduced cells (Fig. 2A). An overrepresentation of RNA-seq reads in the rhlI 5' UTR can also be found in previously published RNA-seq data sets of wild-type P. aeruginosa strain PA14 (19, 33). We also identified an RNA 3' end in the antisense orientation to the rhlI ORF overlapping the sequence of rhlI, although expression of this RNA appeared to be very low (Fig. 3A and see Fig. 6B below).

The 5' UTR does not appear to encode a C4-HSL riboswitch. Because term-seq has been used to discover 5' UTR-derived ribo-regulators that mediate premature transcription termination in other bacteria (26), we asked whether the rhlI 5' UTR might code for a C4-HSL-responsive riboswitch. We first approached this question by using bioinformatics. Neither the PASIFIC (34) nor RFAM (35) predictive structure analysis programs revealed any putative riboswitch-like motifs in the rhlI 5' UTR. While informative, these searches are not exhaustive; therefore, we also addressed this question experimentally. We constructed an E. coli reporter containing the entire rhlI 5' UTR through the first 30 codons fused to lacZ. Expression of this construct was arabinoinducible. We found that when expression of the construct was activated with arabinose, there was no effect on β-galactosidase levels when C4-HSL was added (see Fig. S1 in the supplemental material). The results from both the bioinformatics and experimental approaches were inconsistent with the idea that the rhlI 5' UTR is a C4-HSL-responsive riboswitch. We cannot rule out the possibility that this UTR may be responsive to other signaling molecules. However, these results led us to test other possible consequences of early rhlI transcription termination.
The RhlS sRNA Regulates Quorum Sensing in *P. aeruginosa*

The 5′ UTR of *rhlI* encodes an sRNA. We hypothesized the premature termination of the *rhlI* 5′ UTR with added AHLs could generate a stable sRNA and investigated this using Northern blot analysis. As shown in Fig. 2B, we detected a QS signal-induced sRNA that is less than 100 nucleotides in length as early as 10 min after exposure to AHLs. This sRNA accumulated for at least 60 min after AHLs were added to the cells. We did not detect the sRNA when we analyzed a 5′ UTR-*rhlI* deletion mutant, consistent with the conclusion that the sRNA is specific to the *rhlI* locus (Fig. 2B). We have named this sRNA RhlS (RhlI-associated sRNA). The location and size of RhlS are consistent with a previously identified 70-nucleotide *P. aeruginosa* sRNA (SPA104) that was not known to be AHL induced (28).

We next mapped the 5′ end of RhlS by primer extension (see Fig. S2A in the supplemental material) and the 5′ end of both RhlS and *rhlI* with 5′ RACE (rapid amplification of cDNA ends) (Fig. 3A; Fig. S2B). We found the predominant transcript start site of both RhlS and *rhlI* corresponded to the +1 position of *rhlI* transcription previously reported (36). We also identified a stem-loop structure followed by a polyuridine tract, consistent with a Rho-independent transcriptional terminator sequence immediately upstream of the RNA 3′ end detected in the *rhlI* 5′ UTR sequence (Fig. 3A). By using the boundaries defined by the transcript start site and term-seq, we infer that RhlS is about 70 nucleotides in length. A transcript of this size is consistent
with the RhlS band seen by Northern analysis (Fig. 2B). Our data suggest that, in the presence of AHLs, the rhl locus can be transcribed from a single rhl promoter into two isoforms: the long isoform encoding full-length rhl mRNA and, as our RNA-seq data suggest, a more abundant short RhlS resulting from premature transcription termination within the 5’ UTR (Fig. 2A).

**RhlS is regulated by QS and is dependent on Hfq.** We next examined RhlS production in wild-type *P. aeruginosa*. Northern blotting showed that RhlS levels in early-logarithmic-phase cells were relatively low, increased in late logarithmic phase, and were at maximal levels in stationary-phase cells (Fig. 3B). There is a LasR binding site in the promoter region of rhl, and rhl is activated strongly by LasR and weakly by RhlR (36). Because the transcript starts for RhlS and rhl appeared to be the same, we hypothesized that RhlS transcription would be activated primarily by LasR and to a lesser extent by RhlR. To test this hypothesis, we monitored RhlS levels in strains deleted for lasR and rhlR by Northern blotting. As predicted, RhlS levels in the LasR mutant were very low and RhlS was modestly decreased in the RhlR mutant (Fig. 3C). Thus, the increase in RhlS as a function of growth appears to be primarily a consequence of LasR-dependent QS induction.

A previous report showed that there was a marginal effect of Hfq on rhl mRNA levels (17). We asked whether Hfq might affect RhlS and the rhl transcript differently. In fact, Northern blotting showed very low levels of RhlS in an Hfq mutant in comparison to levels in wild-type cells (Fig. 3D). This is consistent with the conclusion that the two transcripts produced from the rhl locus have different requirements for Hfq. RhlS levels are drastically altered in the absence of Hfq, while rhl levels are only slightly altered (~1.5-fold decreased in an hfq mutant) (17).

**Disruption of the RhlS terminator reduces C4-HSL production.** From the term-seq analysis, we estimate that RhlS is the predominant transcript derived from the rhl promoter, with full-length rhl mRNA accounting for between 3 and 15% of the total at steady state. RNA structure prediction using Mfold (37) showed a predicted structure with a 5’ end hairpin and a Rho-independent terminator (Fig. 4A). We hypothesized that disruption of the Rho-independent terminator should result in increased transcriptional readthrough producing more full-length rhl transcript and thus elevated levels of C4-HSL. We tested this hypothesis by constructing a strain where the Rho-independent terminator is deleted (RhlS-Δterm). Surprisingly, the amount of rhl mRNA in this mutant was similar to that in wild-type cells (see Fig. S3 in the supplemental material), but RhlS levels were lower (Fig. 3B). The deletion may have eliminated an essential Hfq binding site present in the terminator (38). The terminator deletion mutant also produced almost 10-fold less C4-HSL than the wild-type (Fig. 4B). Because the wild type and mutant had roughly equivalent levels of rhl mRNA, we surmise that the defect in C4-HSL production in the terminator mutant is due to RhlS-mediated posttranscriptional regulation of rhl.

To gain insight into whether the terminator sequence or structure of RhlS was responsible for the phenotype exhibited by the terminator deletion mutant, we made point mutations in the RhlS sequence. These point mutations (Fig. 4A, Mut A) should disrupt the terminator structure while leaving the sequence largely intact. We also made a compensatory mutation that should restore base pairing in the terminator and thus recover the structure (Fig. 4A, Mut A+B). The structure-disrupting mutant and the compensating mutant had similar levels of rhl mRNA but produced low levels of C4-HSL and low levels of RhlS (Fig. 4B to D). These data are consistent with the conclusion that the sequence of the RNA in the RhlS terminator region is essential for RhlS function, and RhlS is important for C4-HSL production but not rhl mRNA levels. It is conceivable that Hfq binds to RhlS in this region to promote the sRNA-mediated posttranscriptional regulation of rhl.

The defect in C4-HSL production can be partially restored by trans-complementation of RhlS on a multicopy plasmid (pRhlS) in the RhlS terminator structure mutation strain (Fig. 4E). Arabinose-induced *P. aeruginosa* (pRhlS) had RhlS transcript levels similar to
FIG 4  RhlS regulation of Rhl translation and C4-HSL production. (A) Predicted Mfold (37) structure of the RhlS sRNA. Gray boxes indicate positions and red letters indicate nucleotides changed for MutA, MutA+B and MutC point mutants. Cyan brackets indicate nucleotides deleted in the RhlS-∆term mutant. (B) C4-HSL levels in RhlS point mutant strains compared to wild type. Supernatant was collected from overnight cultures of WT PAO1 (MPK0409) and the isogenic RhlS-∆term mutant (MPK0555), MutA mutant (MPK0576), MutA+B mutant (MPK0619), and as a control, the PAO1 ΔlasI ΔrhlI mutant (MPK0493) after 24 h. C4-HSL was extracted and measured by using the C4-HSL bioassay. Values are the means of three biological and two technical replicates, and error bars are standard deviations. (C) Levels of rhI mRNA in the wild-type PAO1, MutA, and MutA+B strains was determined by quantitative reverse transcription-PCR (qRT-PCR) using primers specific to the rhI open reading frame. The amount of rhI mRNA was calculated with a standard curve and normalized to levels of the housekeeping control gene groEL. Values are the mean of two biological and two technical replicates. Bars are standard deviations. (D) Levels of RhlS in the point mutant strains. The wild type and the isogenic MutA and MutA+B mutants were grown and Northern analysis performed as in Fig. 3. (E) RhlS can partially complement the MutA C4-HSL production defect when provided in trans. Wild-type PAO1 and the PAO1 MutA point mutant were transformed with the empty pMKT1 vector or pRhlS. Single colonies were inoculated into LB plus 50 mM MOPS and grown in the presence or absence of 1% L-arabinose at 37°C. After 24 h, C4-HSL was extracted and measured. Results are means of three biological and two technical replicates, and bars are the standard deviation. (F) Western blot analysis of VSV-G epitope-tagged RhlI. Strains: WT-RhlI-VSV-G (MPK0698), MutA-VSV-G (MPK0689), MutA+B-VSV-G (MPK0697), asRhlS-1-VSV-G (MPK0687), and ΔrhlI (MPK0627). Cells were grown as in panel B. RhlI levels were normalized to the corresponding RNA polymerase band and are presented as a percentage of wild type. The image is representative of three independent experiments.
those of wild-type cells indicating expression from the pRhlS plasmid is physiologically relevant (see Fig. 5A in the supplemental material). Additionally, when an arabinose-induced promoter-rhl fusion was integrated in the Escherichia coli chromosome, the cells produced micromolar amounts of C4-HSL similar to those of wild-type P. aeruginosa (Fig. 5B). This indicates the mechanism by which RhlS induces rhl translation is either conserved between E. coli and P. aeruginosa or contained entirely within the RhlS-rhl locus. Our data suggest that high levels of RhlS are required to maintain wild-type levels of C4-HSL but are not required for rhl mRNA accumulation. In our experiments, rhl mRNA levels remain unchanged regardless of RhlS expression, while C4-HSL levels vary in mutants expressing less RhlS.

RhlS controls translation of rhl mRNA. Because mutants that produce normal levels of rhl mRNA but low levels of RhlS produce low levels of C4-HSL, we hypothesized that RhlS might stimulate translation of rhl mRNA. To test this hypothesis, we incorporated a C-terminal vesicular stomatitis virus glycoprotein G (VSV-G) epitope tag at the native rhl locus such that it encoded an RhlI-VSV-G polypeptide. We examined levels of this tagged RhlI in our RhlS mutant strains by Western blotting (Fig. 4F). Consistent with the hypothesis, RhlI levels were lower in the RhlS mutants than in the wild type. We confirmed that Rhl-VSV-G was active by showing the Rhl-VSV-G-tagged version produced C4-HSL levels comparable to those of the native RhlI protein (see Fig. S5 in the supplemental material). Thus, RhlS appears to affect translation but not the rhl mRNA.

Direct regulation of fpvA by RhlS. It seemed possible that RhlS might affect translation of genes other than rhl. To assess this possibility, we used the base-pairing prediction algorithm TargetRNA2 (39) to search for genomic regions that might base pair with RhlS (Table S1, tab C). The hit with the most extensive base-pairing complementarity was a 16 nucleotide region in the first five codons of the fpvA open reading frame that pairs with RhlS (Fig. 5A). The fpvA gene product is a receptor for the P. aeruginosa siderophore pyoverdine (27).

To test the hypothesis that RhlS controls fpvA expression, we created an arabinose-inducible translational reporter containing the fpvA 5' UTR through the first 25 codons of the fpvA ORF fused in frame to lacZ and placed this construct on the chromosome of E. coli. In this construct, we either expressed RhlS on an arabinose-inducible plasmid (pRhlS) or included the empty vector (pMKT1). Levels of β-galactosidase in arabinose-grown cells containing pRhlS were about 25% of the levels in cells without RhlS (Fig. 5B). To test whether the RhlS repression of fpvA was by direct base pairing via the 16-nucleotide region identified in the TargetRNA2 analysis, we did the following: We first changed the RhlS sequence to disrupt base pairing with fpvA. When we used a plasmid (pMutC) expressing the mutant RhlS in place of wild-type RhlS, fpvA-lacZ expression was not repressed (Fig. 5B). We then constructed an arabinose-inducible fpvA-lacZ reporter with a mutation that compensated for the pMutC mutation, and this restored lacZ repression (Fig. 5C). We note that levels of fpvA-lacZ expression in cells containing the compensatory mutation are lower than those in cells containing the wild-type fpvA-lacZ fusion. We believe this may be due to the fact that changing the sequence of the first few fpvA codons decreased translation efficiency. These experiments indicate that RhlS can serve to regulate fpvA mRNA translation by a direct base-pairing mechanism, and they point to a link between QS and iron homeostasis in P. aeruginosa.

The rhl antisense RNA may be involved in regulating rhl translation. As mentioned earlier, we detected low levels of an rhl antisense RNA in our term-seq analysis (Fig. 3A). A map showing the chromosomal region encoding this asRNA with the term-seq detected TTS is shown in Fig. 6A. We confirmed the existence of the antisense RNA, which we call asRhlS, by Northern blot analysis. Expression of asRhlS was at peak, although still low, abundance during logarithmic growth of P. aeruginosa (Fig. 6B). The size of the asRhlS band is slightly less than 200 nucleotides. Because asRhlS is in low abundance, we were unable to map the exact transcription start site by
However, based on the TTS determined by our term-seq analysis and the size of the band on the Northern blot, we identified a putative TSS (transcription start site) for asRhlS (Fig. 6A; see Fig. S6 in the supplemental material). We predict asRhlS overlaps the beginning of the \textit{rhlI} ORF as well as the entire \textit{rhlI} 5' UTR. Previous transcription start site mapping identified an antisense TSS in strain \textit{P. aeruginosa} PA14 in close proximity (Fig. 6A), 40 nucleotides upstream of our putative TSS (19).

We could not identify a \( \sigma^{70} \) promoter-like −10 and −35 region for either the putative PAO1 asRhlS +1 or the known PA14 +1, but we did identify a possible promoter consistent with a \textit{P. aeruginosa} extracytoplasmic function (ECF) \( \sigma^{E} \) consensus (Fig. 6A) upstream of the putative TSS of the asRhlS in PAO1 (40). Surprisingly, disrupting this sequence increased asRhlS expression (Fig. 6B), leading to decreased C4-HSL (Fig. 6C) and about 25% less RhlI protein compared to the wild type (Fig. 4F).

\textit{P. aeruginosa} has 19 ECF sigma factors (reviewed in reference 41). We do not know which of these might be involved in asRhlS induction, and we leave it to future studies to elucidate whether and how asRhlS regulates \textit{rhlI}.

**DISCUSSION**

By term-seq mapping of \textit{P. aeruginosa} TTSs in a QS AHL signal synthesis mutant with or without added AHLs, we identified a number of QS-regulated sRNAs. Our list includes seven AHL-regulated sRNAs, all of which had been detected previously but never reported to be associated with QS (Table 1). Three of these sRNAs were highly induced by AHLs (Table 1, Fig. 1C and D, and Fig. 2A) while the other four showed weaker
induction by AHLs. In our previous high-resolution RNA-seq analysis of *P. aeruginosa*, we described two LasR-activated sRNAs, Lrs1 and Lrs2 (19), which were not identified in our term-seq analysis. We stress that our analysis was not an exhaustive mapping of QS-dependent sRNA expression. We analyzed only one *P. aeruginosa* strain grown under one condition, 60 min following exposure to AHLs. Furthermore, we used stringent requirements to call a TTS. However, our analysis opens an avenue for future discovery of *P. aeruginosa* QS-dependent sRNA expression.

Here we focused on the most highly AHL-induced sRNA under our conditions, which we have called RhlS. RhlS is encoded in the 5’ UTR of the C4-HSL synthesis *rhlI* gene, it is 70 nucleotides in length, it appears to require Hfq, it stimulates *rhlI* mRNA translation in a trans-acting fashion, and it interferes with the posttranscriptional regulation of an unlinked gene, which codes for the pyoverdine receptor FpvA. Although RhlS was induced when we added both 3OC12-HSL and C4-HSL to growing cells, we presume that induction is primarily a response to 3OC12-HSL because RhlS expression showed a strong dependence on the 3OC12-HSL receptor LasR and only a weak dependence on the C4-HSL receptor RhlR.

**FIG 6** An antisense RNA is encoded in the *rhlI* locus and regulates C4-HSL production. (A) Schematic and sequence of the asRhlS promoter region, including the overlap with RhlS and *rhlI*. Blue boxes indicate the putative +1 of transcription for PA01 and the known +1 for PA14 and red box indicates the asRhlS termination point. The dashed line indicates a predicted -1 site for asRhlS and the consensus sequence is indicated above. Red letters are the nucleotide changes for the asRhlS-1 mutant. For reference and orientation the *rhlI* start codon, *rhlS* start and stop, and *rhlR* stop codon are indicated. The ribosome binding site (RBS) for *rhlI* is shaded gray. (B) The asRhlS-1 mutation increases expression of asRhlS. WT PAO1 and the isogenic asRhlS-1 promoter mutant (MPK0637) were grown and processed for Northern analysis as in Fig. 3 with an oligonucleotide specific to asRhlS. * indicates the wild-type asRhlS transcript band. (C) C4-HSL levels are reduced in the asRhlS-1 promoter strain. Wild-type PAO1 (MPK0409) and asRhlS-1 (MPK0637) were grown and C4-HSL levels were determined as in Fig. 4. Values are means of three biological and two technical replicates for each strain, and error bars are standard deviations. ** indicates *P < 0.005 using an unpaired t test with Welch’s correction.
The RhlS and rhlI transcript start sites appear to be one and the same: There are two RNA isoforms produced from this transcription start site, the shorter RhlS and the longer rhlS-rhlI isoforms. Some sRNAs can repress premature transcription termination within a 5′ UTR by binding and inhibiting Rho-dependent termination (42). However, the presence of a Rho-independent terminator at the 3′ end of RhlS (Fig. 3A and 4A) suggests that the long rhlS-rhlI isoform might result from leaky or imperfect Rho-independent transcription termination rather than by inhibition of Rho-dependent termination. Whether the efficiency of rhlS-rhlI transcription termination changes upon different growth or stress conditions, as described previously for the E. coli SgrS and RyhB sRNAs (43), is unclear at this point. However, similar instances of transcriptional readthrough have been shown in other bacteria. For example, in Salmonella, leaky transcriptional readthrough of the IrsK sRNA terminator leads to a long IrsK-orf45-anrP transcript and a stable short IrsK sRNA, which can then act in trans to increase translation of orf45 and anrP (44). Similarly, incomplete transcription termination of the Salmonella gltIJKL operon, which codes for a glutamate-aspartate transporter, produces either the long gltIJKL mRNA or a short gltI mRNA from which the Hfq-dependent SroC sRNA is processed (45). The SroC sRNA then acts as a sponge to relieve repression of the gltIJKL operon. For reasons discussed below, we hypothesize that RhlS may be functioning to relieve interference with rhlI mRNA translation perhaps by titration of the asRhlS by a SroC sponge-like mechanism.

We find it interesting that the RhlS isoform but not the full-length isoform required Hfq for their function. It is not surprising that the long isoform does not require Hfq, as rhlI transcript levels are minimally altered in an Hfq mutant (17). This sort of differential response of an sRNA and a longer RNA containing the sRNA sequence to Hfq is not unique to RhlS and rhlI. Recently it was shown in E. coli the Rho-independent terminators in the Hfq dependent sRNAs SgrS and RyhB allowed transcriptional readthrough, which produces longer Hfq-independent transcripts (43).

Previous work showed that despite minimal changes in the rhlI mRNA in the absence of Hfq, translation of rhlI and C4-HSL production were reduced in an Hfq mutant (17). We can now explain these reductions. We show that Hfq is important for RhlS, and RhlS is required for normal rhlI translation and thus C4-HSL production. We note that regulation of rhlI is complex and also affected by sRNAs other than RhlS. The RNA binding protein RsmA, which binds GGA motifs in the loops of RNA hairpins to repress translation, was shown to repress rhlI translation and C4-HSL production (46). Additionally, the sRNA RsmY, which binds to RsmA to relieve translational repression, has also been implicated in rhlI regulation and C4-HSL production by an indirect mechanism involving Hfq stabilization of RsmY (17). Although several GGA motifs are present in RhlS, most are not present in the predicted hairpin loops (although one GGA motif is partially buried), suggesting RhlS likely does not affect translation of rhlI through titration of RsmA.

We show that RhlS can function to stimulate rhlI translation in a trans-acting fashion. RhlS expressed from a multicopy plasmid (at physiologically relevant levels) restores C4-HSL levels almost to those of wild-type PA01 (Fig. 4E; Fig. 5A). This finding rules out the possibility the regulation is due to inherent factors in the rhlS-rhlI transcript itself (e.g., RNA structure alters transcription or translation efficiency of rhlI). What is unclear at this point is how RhlS mediates this positive regulation of rhlI. We have two models for how this RhlS regulation may occur. First RhlS could act directly on rhlI to relieve translational repression mediated by a highly structured 5′ UTR. Mfold predicts the secondary structure of the rhlI 5′ UTR occludes the primary RBS by a stem-loop structure (see Fig. S7 in the supplemental material). It is possible RhlS activates rhlI in trans by base pairing to sequences opposite the rhlI RBS, relieving occlusion and facilitating translation. Examples of this type of regulation exist in Pseudomonas (and other bacteria) where an sRNA base pairs to a highly structured 5′ UTR to relieve translation repression mediated by RBS occlusion (13, 47–49). Alternatively, as we propose below, RhlS could act in trans by a sponge-like mechanism to sequester the asRhlS that appears to repress rhlI translation.
Our term-seq and RNA-seq analyses also uncovered the antisense RNA asRhlS. The existence of this RNA was confirmed by Northern blotting, and the Northern blotting also revealed that while expression of the asRhlS is low compared to that of RhlS, it is detectable in early-logarithmic-phase cells and not in stationary-phase cells (Fig. 6B). The asRhlS overlaps the beginning of the rhlI ORF, RBS, and the 5’ UTR of rhlI. It is possible that asRhlS base pairs with the rhlI mRNA to block translation. Given these data, it is possible that RhlS acts as an asRhlS sponge due to the extensive predicted complementarity between RhlS and asRhlS. Under this hypothesis, this potential interaction between RhlS and asRhlS would sequester asRhlS and relieve the translational repression of rhlI. Although this is an intriguing hypothesis, the relationship between RhlS and asRhlS requires further investigation.

Finally, besides having a role in rhlI autoregulation, we identified a region of RhlS complementarity in the mRNA of the pyoverdine receptor gene fpvA. By analyzing the influence of mutations in RhlS and compensatory mutations in the fpvA 5’ UTR on a fpvA-lacZ translational fusion we showed that RhlS interferes with translation of fpvA via a direct base-pairing mechanism. We have not investigated the physiological significance of this interaction, nor have we searched exhaustively for other potential RhlS-regulated mRNAs; however, we have provided our TargetRNA2 list as a resource for the community (Table S1, tab C). We have thus described an additional layer of gene regulation in the intricate P. aeruginosa quorum sensing circuitry.

MATERIALS AND METHODS
Details of additional materials and methods are provided in Text S1.

Bacteria and growth conditions. The bacterial strains, plasmids, and oligonucleotides used in this study are described in Table S1, tab D. Details of strain construction and experimental growth conditions are listed in the Text S1.

RNA extraction, library preparation and sequencing. For RNA-seq, the RNA was extracted using TRIzol and phenol-chloroform. Whole transcriptome RNA-seq libraries and term-seq libraries were prepared as described previously (26).

RNA extraction for Northern blotting. RNA extraction for northern analysis was performed by hot acid phenol-chloroform extraction as described previously with minor changes (see Text S1) (50).

Northern blot analysis. Northern blotting was performed as described previously (50) with minor modifications. Briefly, RNA was separated on 8% polyacrylamide–6 M urea gel (National Diagnostics) and transferred to a Hybond-XL membrane (GE Healthcare). Membranes were probed with [32P]ATP end-labeled oligonucleotides specific to the desired transcript (Table S1, tab D) and exposed to Amersham Hyperfilm MP (GE-Healthcare) at –80°C.

C4-HSL and β-galactosidase measurements. C4-HSL was ethyl acetate extracted from 24-h LB plus 50 mM MOPS (morpholinepropanesulfonic acid) culture supernatant as described previously (51). The amount of C4-HSL was determined by using an E. coli (pECP61.5) bioassay (52, 53) and the Tropix Galacto-Light Plus reagent (Invitrogen).

Data availability. RNA-seq and term-seq data sets have been deposited in the European Nucleotide Database (ENA) under study accession no. PRJEB31965.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02253-19.

TEXT S1, PDF file, 0.3 MB.
FIG S1, PDF file, 0.2 MB.
FIG S2, PDF file, 0.2 MB.
FIG S3, PDF file, 0.3 MB.
FIG S4, PDF file, 0.1 MB.
FIG S5, PDF file, 0.1 MB.
FIG S6, PDF file, 0.2 MB.
FIG S7, PDF file, 0.2 MB.
TABLE S1, XLSX file, 0.6 MB.

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