# **RESEARCH ARTICLE**



# The Pseudomonas aeruginosa Two-Component Regulator AlgR Directly Activates rsmA Expression in a Phosphorylation-Independent Manner

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ABSTRACT Pseudomonas aeruginosa is an important pathogen of the immunocompromised, causing both acute and chronic infections. In cystic fibrosis (CF) patients, P. aeruginosa causes chronic disease. The impressive sensory network of P. aeruginosa allows the bacterium to sense and respond to a variety of stimuli found in diverse environments. Transcriptional regulators, including alternative sigma factors and response regulators, integrate signals changing gene expression, allowing P. aeruginosa to cause infection. The two-component transcriptional regulator AlgR is important in P. aeruginosa pathogenesis in both acute and chronic infections. In chronic infections, AlgR and the alternative sigma factor AlgU activate the genes responsible for alginate production. Previous work demonstrated that AlgU controls rsmA expression. RsmA is a posttranscriptional regulator that is antagonized by two small RNAs, RsmY and RsmZ. In this work, we demonstrate that AlgR directly activates rsmA expression from the same promoter as AlgU. In addition, phosphorylation was not necessary for AlgR activation of *rsmA* using *algR* and *algZ* mutant strains. AlgU and AlgR appear to affect the antagonizing small RNAs rsmY and rsmZ indirectly. RsmA was active in a mucA22 mutant strain using leader fusions of two RsmA targets, tssA1 and hcnA. AlgU and AlgR were necessary for posttranscriptional regulation of tssA1 and hcnA. Altogether, our work demonstrates that the alginate regulators AlgU and AlgR are important in the control of the RsmA posttranscriptional regulatory system. These findings suggest that RsmA plays an unknown role in mucoid strains due to AlgU and AlgR activities.

**IMPORTANCE** *P. aeruginosa* infections are difficult to treat and frequently cause significant mortality in CF patients. Understanding the mechanisms of persistence is important. Our work has demonstrated that the alginate regulatory system also significantly impacts the posttranscriptional regulator system RsmA/Y/Z. We demonstrate that AlgR directly activates *rsmA* expression, and this impacts the RsmA regulon. This leads to the possibility that the RsmA/Y/Z system plays a role in helping *P. aeruginosa* persist during chronic infection. In addition, this furthers our understanding of the reach of the alginate regulators AlgU and AlgR.

**KEYWORDS** *P. aeruginosa*, RsmA, AlgR, mucoid, *Pseudomonas aeruginosa*, cystic fibrosis, *mucA*, two-component regulatory systems

The opportunistic pathogen *Pseudomonas aeruginosa* possesses multiple virulence factors for causing disease. This allows *P. aeruginosa* to cause both acute and chronic infections. Acute infecting strains are characterized by the presence of type IV pili (T4P), flagella, and a type III secretion system (T3SS) (1–3). In contrast, chronic infecting strains diversify (4, 5) and frequently do not express T3SS, T4P, or flagella (6, 7). Chronic infecting strains often form biofilms composed of exopolysaccharides, such as alginate, and signal a decline in lung function in CF patients (8–11). Alginate

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biosynthesis requires activation of the alternative sigma factor AlgU (12, 13) and the two-component transcriptional regulator AlgR (12, 14, 15).

Two-component signal transduction systems are important in regulating the bacterial response to environmental conditions. AlgR controls both acute and chronic virulence genes (16–18). In the case of acute infections, AlgZ phosphorylates AlgR and activates the *fimU* operon encoding minor pilins important in pilus biogenesis (19–21). The production of alginate, indicative of chronic infection, does not require AlgR phosphorylation (22). This fact has been demonstrated by deletion of the histidine sensor kinase gene *algZ* (23) and by the expression of an *algR* mutant allele encoding an asparagine in place of the conserved aspartate (22).

In chronic *P. aeruginosa* infections, the bacteria frequently acquire spontaneous *mucA* mutations that free the alternative sigma factor AlgU (12, 14, 24–26). AlgU and AlgR regulate the alginate biosynthesis genes producing mucoid colonies (12). In addition, AlgR decreases the expression of acute virulence factors, such as the T3SS in chronic infections when *mucA* mutations occur (7, 27). Therefore, AlgR has multiple roles depending on the type of infection and the phosphorylation state of AlgR: phosphorylated in acute infections activating the *fimU* operon and unphosphorylated in chronic infections activating alginate biosynthesis.

Previous work implicated the AlgZ/R system in the control of *rsmA* expression (28), but a mechanism for AlgR activation of *rsmA* was not investigated. RsmA is considered a global regulator that controls the expression of many *P. aeruginosa* genes by binding to mRNAs (29). Two noncoding RNAs, RsmY and RsmZ, counteract RsmA (29, 30). In this study, we sought to determine how AlgR activates *rsmA* and further investigate the role of RsmA in *P. aeruginosa* strains containing an *mucA* mutation. We further examined *rsmY* and *rsmZ* expression in *algR* mutant strains.

We demonstrate that AlgR directly activates *rsmA* expression in a phosphorylationindependent manner and that both AlgR and AlgU are important in activating *rsmA* expression in mucoid strains. The activity of RsmA is not thought to be significant in chronic infecting strains, such as *mucA* mutants. However, we also provide evidence that RsmA is active in mucoid *P. aeruginosa*. Altogether, our work shows that the two-component regulator AlgR can affect gene expression through RsmA, providing another mechanism for how AlgR impacts virulence gene regulation. We postulate that AlgR and RsmA likely function as a rheostat, as opposed to a switch, and suggest that RsmA plays a role in chronic infections.

### RESULTS

*mucA* mutant strains require AlgR for increased *rsmA* expression. Previous studies suggested that AlgU and AlgR control *rsmA* expression (28, 31). However, whether both AlgZ and AlgR were involved in the control of *rsmA* expression was not tested. We constructed and assayed an *rsmA* transcriptional fusion (Fig. 1A) that contains both *rsmA* promoters (rsmATF1-*lacZ*). The rsmATF1-*lacZ* fusion was analyzed in the wild-type strain *P. aeruginosa* PAO1,  $\Delta algR$  and algZ mutants, and in the corresponding *mucA22* mutants. The *algZ* mutant has a mutation in the conserved histidine residue, which prevents AlgZ-mediated phosphorylation of AlgR (19, 32) but does not disrupt the internal *algR* promoter (33). As shown in Fig. 1B, there was a slight increase in rsmATF1-*lacZ* activity in a  $\Delta algR$  mutant. When the rsmATF1-*lacZ* fusion was assayed in an *algZ* mutant strain, there was also a slight increase in reporter activity (Fig. 1B). The modest increase in reporter activity suggests that AlgZ and AlgR have a minor role in *rsmA* regulation in strain PAO1.

As previous studies also indicated a role for AlgU in regulating *rsmA* expression, the rsmATF1-*lacZ* transcriptional fusion was assayed in a *mucA22* strain, where AlgU is most active. Reporter activity was increased ~3-fold in a *mucA22* strain, as previously described (31) (Fig. 1B). However, in the *mucA22*  $\Delta algR$  double mutant, there was a drastic decrease (~3-fold) in rsmATF1-*lacZ* activity (Fig. 1B). The *mucA22* algZ mutant strain had rsmATF1-*lacZ* activity almost identical to that of the *mucA22* mutant strain



**FIG 1** AlgR, but not AlgZ, is necessary for increased *rsmA* expression in mucoid strains. (A) Schematic of *rsmA* genomic region. The sequence below the genomic schematic indicates the AlgU-dependent promoter region. Underlined sequences are potential AlgR-binding sites. The bent arrow and bold nucleotide indicate the transcriptional start sites. The *rsmA* transcriptional fusion rsmATF1-*lacZ* used in this study is listed below. Arrows indicate the primers used and the numbers indicate the primer locations relative to the translational start site. (B) The transcriptional fusion rsmATF1-*lacZ* (Fig. 1A) was assayed in the indicated strains after growth for 8 h in LB broth. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.01 (\*) and <0.0001 (\*\*\*). (C) Western blot analysis of the indicated strains containing an HA-tagged *rsmA* allele. PAO1 without the *rsmAHA* allele was run as a negative control. Densitometry analysis was performed using a duplicate gel and staining for total protein using Coomassie blue, and all strains were normalized to PAO1 containing the *rsmAHA* allele. Western blotting was performed four times, and densitometry analysis is indicated below the Western blot. A representative Western blot is shown above the densitometry. A one-way ANOVA with Tukey's posttest was used to determine statistical significance. \*\*, P < 0.001; \*\*\*, P < 0.0001.

(Fig. 1B). These data implicate AlgR but not AlgZ in *rsmA* regulation in a *mucA22* mutant strain and suggest that AlgR phosphorylation is not necessary for *rsmA* activation.

To confirm that AlgR affected *rsmA* expression, an epitope-tagged *rsmA* allele was introduced into the wild-type strain (PAO1), *mucA22* mutant, and respective *algR* mutant strains and analyzed by Western blot analysis. As shown in Fig. 1C, a slight decrease in RsmA levels in the  $\Delta algR$  mutant strain was detected compared to the wild-type strain PAO1. As reported previously (31), a *mucA22* mutant strain had drastically increased RsmA levels (Fig. 1C). The *mucA22*  $\Delta algR$  mutant strain had significantly decreased RsmA compared with the *mucA22* mutant strain (Fig. 1C). The Western blot analysis confirmed the transcriptional fusion analysis and supported a significant role for AlgR activating *rsmA* in the mucoid *mucA22* mutant strain but not in the nonmucoid strain PAO1.

**AlgR phosphorylation is not required for activation of** *rsmA* **expression.** To further investigate the role of AlgR phosphorylation in the regulation of *rsmA*, *algR* site-directed mutants were constructed that mimic either the unphosphorylated (D54N) or phosphorylated form of AlgR (D54E). The *algR*D54N and *algR*D54E mutant alleles were also constructed in the *mucA22* mutant background to confirm a phosphorylation-independent mode of AlgR activation of *rsmA*. Both the *mucA22 algR*D54N and the *mucA22 algR*D54E mutant strains were mucoid (data not shown), supporting the notion that phosphorylation is not necessary for alginate production and that the mutant AlgR proteins produced in these strains were still functional. The *mucA22*D54N mutant had



**FIG 2** AlgR phosphorylation is not required for *rsmA* activation. Mutants containing either a mutated aspartate to asparagine (D54N) or aspartate to glutamate (D54E) were constructed in both the wild-type background PAO1 and the *mucA22* mutant background. The *rsmA* transcriptional fusion rsmATF1-*lacZ* was introduced into the strains indicated, resulting in single-copy chromosomal transcriptional fusions. The indicated strains were grown for 8 h in LB broth and assayed for  $\beta$ -galactosidase activity. Values indicate the actual  $\beta$ -galactosidase activity minus the vector control (-28 Miller units). Differences from the wild-type strain PAO1 or *mucA22* mutant were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001.

increased *rsmA* reporter activity compared to a *mucA22* mutant strain (Fig. 2). The *mucA22 algR*D54E strain had decreased rsmATF1-*lacZ* activity compared to the *mucA22* mutant (Fig. 2). Because the *mucA22 algR*D54N and the *mucA22 algZ* mutant strains had elevated and similar levels, respectively, of rsmATF1-*lacZ* activity (Fig. 2 and 1B, respectively) compared to the *mucA22* mutant strain, this suggests that phosphorylation of AlgR is not required for *rsmA* activation.

AlgR regulates the distal *rsmA* promoter. Previous work determined that *rsmA* has two promoters (31). An RNase protection assay was performed using a probe spanning the upstream region of *rsmA* (Fig. 3A) to determine which *rsmA* message AlgR affected. As we previously reported (31), two *rsmA* messages were seen in both the wild-type strain PAO1 and the *mucA22* mutant strain, with the longer transcript increased in the *mucA22* mutant strain (Fig. 3B). There was little difference in the *rsmA* transcripts between PAO1 and the *\Delta algR* mutant (Fig. 3B). In contrast, there was a substantial decrease in the longer *rsmA* transcript in the *mucA22*  $\Delta algR$  mutant strain compared to the *mucA22* parent strain (Fig. 3B). The specificity of the *rsmA* probe was confirmed using the probe incubated with yeast tRNA (+RNase) and using a  $\Delta rsmA$  mutant (Fig. 3B). These results suggest that AlgR is most important for *rsmA* control in the *mucA22* background and that AlgR controls the distal *rsmA* promoter that is also under control by AlgU.

To confirm that AlgR regulates the distal *rsmA* promoter, transcriptional fusions containing individual *rsmA* promoters were used (Fig. 3A; see also Fig. S1A in the supplemental material). The transcriptional fusion rsmATF2-*lacZ* contains only the proximal *rsmA* promoter (Fig. S1A). The transcriptional fusion rsmATF3-*lacZ* (Fig. 3A) contains only the distal promoter that has been shown to be controlled by AlgU (31). The deletion of *algR* had no effect in the rsmATF2-*lacZ* transcriptional fusion in the wild-type or a *mucA22* background (Fig. S1B). The transcriptional fusion rsmATF3-*lacZ* had increased reporter activity in a *mucA22* mutant strain compared to that in PAO1 (Fig. 3C). Compared to PAO1, no change in rsmTF3-*lacZ* activity was seen in a *ΔalgR* mutant strain (Fig. 3C). However, *algR* deletion in a *mucA22* mutant strain (*mucA22 ΔalgR*) resulted in a significant decrease in rsmATF3-*lacZ* activity (Fig. 3C), supporting the RNase protection assay. Taking these results together, we conclude that AlgR activates the distal *rsmA* transcription from the distal promoter.

AlgR directly binds the distal *rsmA* promoter. We hypothesized that AlgR directly activated the distal *rsmA* promoter. In support of this idea, two potential AlgR-binding sites are located upstream of *rsmA*. One of the potential AlgR-binding sites is 2



**FIG 3** AlgR controls the distal AlgU-dependent *rsmA* promoter. (A) Schematic of the RNase protection assay probe used. Numbers indicate distances from the *rsmA* translational start site. The transcriptional fusion rsmATF3-*lacZ* is indicated at the bottom. Arrows indicate the primers used and their location in reference to the *rsmA* translational start site. (B) A representative RNase protection assay performed on the indicated strains after 8 h of growth in LB broth. Total RNA was isolated from each strain and hybridized to 800 pg of biotinylated *rsmA* probe. After treatment with RNase A/RNase T1, protected probe fragments were detected after electrophoresis through a denaturing polyacrylamide gel and transfer to a nylon membrane. Protected probe fragments are indicated to the left. RNase protection assays were performed three times. (C) The *rsmA* transcriptional fusion rsmATF3-*lacZ* was introduced into the indicated strains to generate strains containing single-copy chromosomal fusions. The strains were grown for 8 h in LB broth and assayed for  $\beta$ -galactosidase activity minus the vector control. Differences from the wild-type strain PAO1 or *mucA22* mutant were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. Fusion analysis was performed three times.



FIG 4 AlgR directly binds the AlgU-dependent rsmA promoter and requires both AlgR-binding sites. Purified AlgR was incubated with either biotinvlated PCR products or annealed primers. (A). Schematic of the rsmA genomic region. Below are fragments or annealed primers and the approximate location upstream of rsmA. Promoters are indicated above as bent arrows and are denoted by PAIgU or PRpoD. The arrowheads indicate the approximate location of the putative AlgR-binding sites. (B) Analysis of biotinylated PCR fragments in gel shift studies. The PCR fragment TF1 represents the entire rsmA upstream region (see panel A). Fragments TF2 and TF3 correspond to the separated rsmA promoters (see Fig. 1). TF2 corresponds to the proximal rsmA promoter, and TF3 corresponds to the distal AlgU-dependent promoter. A minus sign indicates probe alone. A plus sign indicates AlgR concentration of 2.5  $\mu$ M. pscF was used as a negative control. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. (C) Titration and competition assay using purified AlgR and the TF3 fragment corresponding to the distal rsmA promoter. The pscF fragment was used as a negative control. TF3 was incubated with different concentrations of AlgR (indicated by the graded triangle; 0.05, 0.25, 1.25, and 2.5  $\mu$ M). TF3 was also used in a competition assay using unlabeled TF3 as a competitive inhibitor. Ratios below indicate the ratio of labeled to unlabeled probe. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. A minus sign indicates probe alone. (D) Two AlgR-binding sites are upstream of the distal rsmA promoter. Annealed primers containing either wild-type or two different mutated AlgR-binding sites were tested in gel shift experiments. The pscF fragment was used as a negative control. The rsmA lanes indicate the probe using annealed primers with both AlgR-binding sites intact. M1 indicates the same as rsmA, except that the furthest upstream AlgR-binding site was mutated. M2 indicates the same as rsmA, except that the further downstream AlgR-binding site is mutated. Shaded arrowheads indicate shifted fragments. White arrowheads indicate unbound probe. Minus signs indicate probe alone. Plus signs indicate an AlgR concentration of 2.5  $\mu$ M.

nucleotides away from the AlgU -35 consensus promoter (Fig. 4A). Purified AlgR was tested in gel shift studies with biotinylated rsmA promoter fragments (Fig. 4A). A biotinylated pscF gene, a component of the T3SS (34, 35), was used as a negative control and did not shift when incubated with purified AlgR (Fig. 4B to D). A PCR fragment representing the entire rsmA upstream region containing both promoters was dramatically shifted using purified AlgR (Fig. 4B), suggesting that AlgR directly binds to at least one of the AlgR-binding sites upstream of rsmA. To further define the region upstream of rsmA that was bound by AlgR, PCR amplicons that corresponded to the transcriptional fusion constructs containing individual rsmA promoters (Fig. 4A) were biotinylated and tested via gel shift analysis. A biotinylated probe containing the proximal promoter (TF2) and upstream sequence to the more distal transcriptional start site did not shift when incubated with purified AlgR (Fig. 4B). This result suggested that AlgR does not bind the proximal rsmA promoter. As shown in Fig. 4B, AlgR bound the distal promoter sequence fragment, TF3, containing the AlgU-dependent promoter and potential AlgR-binding sites. The TF3 fragment containing the AlgU promoter was tested in a competition assay using increasing concentrations of AlgR and unlabeled

probe. A 1:1 ratio of labeled to unlabeled probe was capable of decreasing the shift of the TF3 probe (Fig. 4C). Competition with the unlabeled specific probe in a 1:5 or 1:10 ratio was able to compete for AlgR binding, as indicated by severe reduction of the probe shift (Fig. 4C). These data suggest that AlgR specifically binds the AlgU-dependent promoter region and strongly suggests that AlgR binds at least one of the AlgR consensus sequences located in this promoter region.

AlgR has previously been shown to bind an 11-bp consensus sequence, with nucleotides 2 to 10 being the most conserved (36). To determine if one, or both, of the putative AlgR-binding sites were required for AlgR binding, we used annealed primers upstream of the distal promoter in gel shift analyses. Approximately 30-bp primers were annealed and biotinylated that contained the predicted AlgR-binding sites. AlgR was able to shift the annealed biotinylated primers containing the two predicted AlgR-binding sites (Fig. 4D). The first putative AlgR-binding site, CCTTTGTC, was mutated, and this mutation resulted in a loss of AlgR binding (Fig. 4D), suggesting that the first AlgR-binding site is required for AlgR binding to the *rsmA* promoter. The second putative AlgR-binding site, CCGTTTGGC, is located 7 bp downstream and directly adjacent to the AlgU – 35 consensus sequence. When the second AlgR-binding site was mutated, AlgR was no longer able to bind to the annealed primers (Fig. 4D). These data suggest that AlgR binds both of the consensus sequences in order to activate *rsmA* expression.

**AlgR regulates** *rsmY* **expression indirectly.** A previous study indicated that *rsmY* and *rsmZ* expression was also increased in a *mucA* mutant background and required AlgR (28). The transcriptional start site of *rsmY* was determined in *Pseudomonas fluorescens* using 5' rapid amplification of cDNA ends (RACE), and this information was used to deduce the transcriptional start site in *P. aeruginosa* (30, 37). To confirm the *P. aeruginosa rsmY* transcriptional start site, we performed primer extension. As indicated in Fig. 5A, a single extension product was obtained after reverse transcription of total RNA that confirmed the *rsmY* transcriptional start site assigned using *P. fluorescens*.

A transcriptional fusion, rsmYTF1-*lacZ* (Fig. 5B), was constructed and assayed in *algR* and *algZ* mutants in both the PAO1 (wild-type) and the *mucA22* background. When a  $\Delta algR$  or *algZ* mutant was tested for *rsmY* reporter activity, there was no decrease compared to PAO1 (Fig. 5C). There was a >3-fold increase in rsmYTF1-*lacZ* activity in a *mucA22* mutant strain compared to PAO1 (Fig. 5C). When tested in a *mucA22*  $\Delta algR$  mutant strain, the increased activity in the *mucA22* mutant strain was reduced to PAO1 levels (Fig. 5C). In the case of the *mucA22 algZ* mutant strain, there was a slight but statistically significant decrease in rsmYTF1-*lacZ* activity (Fig. 5C). Overall, the fusion results suggest that AlgR affects *rsmY* expression, but only in the *mucA22* background.

To confirm that the *rsmY* levels decreased, Northern blotting was performed on the same strains. Two bands hybridized to the *rsmY* probe, as has been seen previously (Fig. 5D) (30, 38). Little difference was seen between a  $\Delta algR$  mutant strain and the wild-type PAO1 (data not shown). However, an increase in both hybridizing RNAs was seen in a *mucA22* mutant strain (Fig. 5D). The *mucA22*  $\Delta algR$  and *mucA22* algZ mutant strains had decreased hybridizing fragments (Fig. 5D). The loading control used (5S rRNA) was consistently uneven upon repeated attempts, but the Northern blotting does confirm the transcriptional fusion analysis. A second probe using *proC* was also used and gave similar results. A  $\Delta rsmY$  strain had no hybridizing RNA, confirming the specificity of the *rsmY* probe. Overall, these results support AlgR control of *rsmY* expression in the *mucA22* strain.

Both *rsmY* and *rsmZ* contain upstream activating sequences (UAS) that GacA controls (39). A potential AlgR-binding site is located 23 nucleotides upstream of the UAS for *rsmY*. An additional AlgR-binding site is present further upstream on the opposite strand (Fig. 5B). Two additional transcriptional fusions, rsmYTF2-*lacZ* and rsmYTF3-*lacZ* (Fig. 5B and S2A), were constructed to determine the sequences required for AlgR control. The rsmYTF2-*lacZ* transcriptional fusion deletes the AlgR-binding sites but retains the UAS sequence (Fig. 5B). When assayed in the *algR* mutant strains in both



FIG 5 AlgR indirectly increases rsmY expression in a mucA22 mutant strain. (A) Primer extension analysis performed on total RNA from strain PAO1. GATC indicates the sequencing ladder. -C is a control without reverse transcriptase. PAO1 is reverse-transcribed PAO1 mRNA. Below is the sequence of the rsmY promoter region. The asterisk denotes the transcriptional start site identified. (B) Schematic of the rsmY genomic region. The UAS is indicated between the numbers from the transcriptional start site. The bent arrows above indicate the primers used for the transcriptional fusions, and the numbers correspond to the distance from the transcriptional start site. P and the bent arrow indicate the start site of transcription. The arrowheads represent potential AlgR-binding sites. The bent arrow below indicates the primer used in the primer extension experiment. Indicated below is the probe generated by in vitro transcription for Northern analysis. Transcriptional fusions are indicated below. Numbers above the arrows indicate the distance from the transcriptional start site. (C) Transcriptional fusion rsmYTF1-lacZ was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for  $\beta$ -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate P values of 0.001 (\*\*) and <0.0001 (\*\*\*). (D) Northern blot analysis of RsmY in the indicated strains after growth for 8 h in LB broth. The  $\Delta rsmY$  mutant was used as a control. The top half is a membrane probed with the RsmY probe, and the bottom portion is a second independent blot of the loading control probed with a 5S rRNA probe. Northern blotting was performed at least three times. (E) Transcriptional fusion rsmYTF2-lacZ was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for  $\beta$ -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate P values of <0.0001. All transcriptional fusion analyses were performed at least three times.



**FIG 6** AlgR increases *rsmZ* expression in a *mucA22* mutant strain indirectly. (A) Primer extension analysis using total RNA isolated from PAO1. GATC indicates the sequencing ladder. -C is a control without reverse transcriptase. PAO1 is reverse-transcribed PAO1 mRNA. Below is the sequence of the *rsmZ* promoter region. The asterisk denotes the transcriptional start site identified. (B) Schematic of the *rsmZ* genomic region. The bent arrow on top indicates the primer used for transcriptional fusion construction. The bent arrow indicated by a "P" indicates the transcriptional start site. The bent arrow below indicates the primer used in primer extension. (C) Transcriptional fusion ZTF1-*lacZ* was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for  $\beta$ -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Asterisks indicate *P* values of 0.001 (\*\*) and <0.0001 (\*\*\*). Transcriptional fusion analysis was performed in triplicate three times. (D) Northern analysis of the indicated strains grown for 8 h in LB broth and total RNA probed with and *rsmZ* probe. A *ΔrsmZ* mutant strain was used to denote the specificity of the probe. A separate blot of the same strains was probed using a 55 rRNA probe (shown below). Northern blotting was performed three times.

PAO1 and *mucA22* backgrounds, a difference was only seen in the *mucA22* background (Fig. 5E). This result suggests that the potential AlgR-binding sites are not necessary for increased *rsmY* reporter activity.

An additional *rsmY* reporter, rsmYTF3-*lacZ*, was constructed that does not contain the UAS sequence. This fusion had very low activity in both PAO1 and *mucA22* (Fig. S2B). This result suggests that the UAS is required for increased *rsmY* reporter activity in a *mucA22* mutant strain as well as PAO1. Overall, the data suggest that AlgR and AlgU indirectly affect *rsmY* expression.

AlgR regulates *rsmZ* expression indirectly. The regulation of *rsmZ* is more complex than *rsmY* and includes regulators other than GacA (38, 40, 41). Primer extension analysis confirmed the location of the transcriptional start site in *P. aeruginosa* that was predicted from *P. fluorescens* (Fig. 6A) (42, 43). A transcriptional fusion, rsmZTF1-*lacZ*, was assayed in the *algZ* and *algR* mutants (Fig. 6B). There was no difference in the *rsmZ* reporter in the  $\Delta algR$  or *algZ* mutant compared to PAO1 (Fig. 6C). There was a 5-fold increase in rsmZTF1-*lacZ* activity in the *mucA22* mutant strain compared to PAO1 (Fig. 6C). In a *mucA22*  $\Delta algR$  mutant strain, there was a significant decrease in rsmZTF1-*lacZ* activity. In a *mucA22 algZ* mutant strain, there was a significant but slight decrease in

rsmZTF1-*lacZ* activity compared to the *mucA22* mutant (Fig. 6C). In all, these results suggest that AlgR plays a major role and AlgZ plays only a minor role, if any, in terms of *rsmZ* expression in the *mucA* mutant background.

To confirm the transcriptional fusion results, we again wanted to monitor the actual RNA levels. Using Northern blot analysis, a trend was seen similar to that in the transcriptional fusions. As shown in Fig. 6D, there were two bands detected, with the larger fragment being the most intense. Previous studies utilizing Northern blotting suggested that the smaller hybridizing fragment represents the small RNA lacking the stem-loop found at the 3' end of the small RNA (30). Therefore, the smaller hybridizing bands may represent the small RNAs lacking a transcriptional terminator. A  $\Delta rsmZ$  mutant was used as a negative control. The *mucA22* mutant strain had increased RsmZ compared to PAO1 (Fig. 6D). The *mucA22*  $\Delta algR$  mutant strain had the most drastic decrease in RsmZ levels, and there was a slight decrease in RsmZ levels in the *mucA22* algR plays a role in increasing the RsmZ small RNA in the *mucA22* background.

We hypothesized that like *rsmY* expression, AlgR indirectly affects *rsmZ* expression. An additional transcriptional fusion, rsmZTF2-*lacZ* (Fig. S3A), was constructed and assayed in the wild-type strain PAO1 and a *mucA22* mutant strain, which lacks the UAS sequence. Both strains had drastically decreased reporter activity (Fig. S3B). Interestingly, the *mucA22* mutant strain had significantly decreased activity compared to PAO1 (Fig. S3B). Further support for an indirect mechanism of AlgR activation was obtained using gel shift analysis of a PCR amplicon of the *rsmZ* upstream region (Fig. S3A), suggesting AlgR does not directly activate *rsmZ* expression. These results suggest that the UAS is required for increased *rsmZ* expression, and we conclude that AlgR affects *rsmZ* expression indirectly.

AlgR is necessary for RsmA activity in a *mucA* mutant background. To ascertain whether AlgR control of *rsmA* was significant in a biological context, the direct RsmA targets *hcnA* and *tssA1* were used to assess RsmA activity (29). A new integrating vector was constructed that contains the *lacUV5* promoter and lacks a ribosome-binding site or a start codon for *lacZ*. When tested in the wild-type strain PAO1, there was no activity of the vector alone.

The hcnA gene encodes part of the hydrogen cyanide synthase enzyme that is implicated in virulence (44, 45). RsmA was shown to previously negatively affect a leader fusion using the tac promoter and the ribosome-binding site region of hcnA (45). An hcnA leader fusion was constructed by annealing 33 nucleotides, including one of the predicted RsmA-binding sites, the ribosome-binding site, and the translational start codon of hcnA. As shown in Fig. 7A, the lacUV5 hcnA-lacZ fusion had activity in strain PAO1. When assayed in a mucA22 mutant strain, there was an  $\sim$ 2.5-fold decrease in reporter expression (Fig. 7A), consistent with increased RsmA levels in the mucA22 mutant strain. If rsmA expression requires AlgR and AlgU, mutating algR and algU should result in increased fusion activity in the mucA22 background due to decreased rsmA expression. Both the mucA22  $\Delta algR$  and the mucA22  $\Delta algU$  mutant strains had at least a 3-fold increase in reporter expression compared to the mucA22 strain (Fig. 7A). The mucA22  $\Delta rsmA$  mutant strain was assayed and had a 3.6-fold increase in reporter activity compared to the mucA22 mutant (Fig. 7A). Overall, these results are consistent with the conclusion that RsmA is active in the mucA22 strain and that AlgU and AlgR are important for the decreased posttranscriptional regulation of hcnA.

Another RsmA target, *tssA1*, was also analyzed. The *tssA1* gene encodes a portion of the T6SS and is directly regulated by RsmA (29, 46). Previous studies have used a *lacUV5 tssA1* construct containing 227 bp of *tssA1* upstream sequence that had extremely low activity (28, 29, 31). However, this fusion was found to contain additional transcriptional controls that resulted in low activity (data not shown). To more carefully assess RsmA activity using *tssA1*, we constructed a new leader fusion by annealing 31 nucleotides, including the predicted RsmA-binding site, the ribosome-binding site, and the trans-



**FIG 7** RsmA is active in a *mucA22* mutant strain. (A) Leader/translational fusion *lacUV5 hcnA-lacZ* (above graph) was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for  $\beta$ -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. (B) Leader/translational fusion *lacUV5 tssA1-lacZ* (above graph) was introduced into the indicated strains in single copy, grown for 8 h in LB broth, and assayed for  $\beta$ -galactosidase activity. Significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. Triple asterisks indicate *P* values of <0.0001. All leader/translational fusions were assayed in triplicate three times.

lational start codon of *tssA1*. The *lacUV5 tssA1-lacZ* fusion had approximately 200 times the activity as the previously constructed *lacUV5 tssA1-lacZ* fusion containing 227 bp of upstream sequence in the wild-type strain PAO1 (Fig. S4). There was no activity when this fusion was assayed in a *mucA22* mutant strain (Fig. 7B and S4). The *lacUV5 tssA1-lacZ* fusion confirmed our previous result (31), demonstrating increased activity in the *mucA22*  $\Delta algU$  mutant strain, validating the use of this construct. When a *mucA22*  $\Delta algR$  mutant was tested, the fusion was also increased from the *mucA22* mutant strain to levels similar to those of the *mucA22*  $\Delta algU$  mutant strain (Fig. 7B). A *mucA22*  $\Delta rsmA$ mutant strain also had statistically significant activity compared to that of the *mucA22* mutant (Fig. 7B). These results demonstrate that AlgR and AlgU are necessary for posttranscriptional regulation of *tssA1*. From these data, we conclude that AlgU and AlgR are both required for increased *rsmA* expression in the *mucA* 



**FIG 8** Model for AlgR activation of *rsmA* and RsmA activity in mucoid strains. In chronic infections, *mucA* mutants arise, leading to increased AlgU activity. In the case of *rsmA*, AlgR and AlgU activate the distal *rsmA* promoter. The dark-shaded triangles indicate the AlgR-binding sites located upstream of *rsmA*. The light-shaded triangle indicates the AlgU-dependent promoter. The model predicts that increased expression of *rsmA* leads to increased RsmA activity, as indicated by the negative regulation of the RsmA targets, *hcnA* and *tssA1*.

and that this leads to posttranscriptional regulation on two known RsmA targets, most likely through RsmA.

# DISCUSSION

AlgR is an important two-component regulator having roles in both acute and chronic infections. In the case of acute infections, AlgR activates the *fimU* operon, enabling the production of T4P (19, 21, 47). In chronic infections, AlgR, AlgU, and other transcriptional regulators activate the production of alginate (22, 48–50). These and other virulence factors in *P. aeruginosa* are often considered mutually exclusive, depending on whether there is an acute or chronic infection (51–53). This work demonstrates that another effect of a *mucA* mutation is that AlgR activity is directed at increasing the posttranscriptional regulator RsmA. AlgR activation of *rsmA* may help explain how AlgR participates in the mutually exclusive production of some virulence factors depending on acute or chronic infection.

The initial description of AlgR as a regulator of rsmA (28) did not evaluate the contribution of AlgR phosphorylation by AlgZ or a mechanism for AlgR activation of rsmA. Because AlgR is part of a two-component system that is important for both acute and chronic infections (19, 32), it was necessary to address the mechanism for AlgR activation of rsmA in order to create a framework to understand how AlgR regulation of rsmA might impact virulence gene expression. AlgR binds a consensus sequence CCGTTCGTC (21, 48, 49), and phosphorylation is thought to enable AlgR to bind potential binding sites that deviate from this consensus, such as the sites found in the fimU promoter (32, 47). However, the rsmA promoter deviates from the AlgR-binding consensus, and AlgR phosphorylation was not required for rsmA expression. This argues against phosphorylation as the sole mechanism for AlgR binding less well-conserved consensus sequences. The studies using the mucA22 mutant strain, the algZ mutant, and the D54N mutant are consistent with the conclusion that AlgR phosphorylation is not required for rsmA activation. As AlgR phosphorylation is not required for alginate expression (22), our results are consistent with the conclusion that AlgR activates rsmA expression in mucoid strains.

The mechanism for AlgR activation of *rsmA* is by directly binding upstream of the AlgU-dependent promoter, further supporting our model (Fig. 8). Gel shift studies determined that two AlgR-binding sites are required for *in vitro* binding. The location of the AlgR-binding sites, the *in vitro* gel shift analysis, transcriptional fusions, and RNase protection assay all support AlgR binding the distal promoter region of *rsmA* and are in accord with the activation of the AlgU-dependent promoter.

Our data suggest that AlgR plays a more important role in regulating *rsmA* expression in *mucA* mutant strains, such as those found in CF patients. If AlgU and AlgR activate *rsmA* expression, as predicted by our model (Fig. 8), this would explain why AlgR did not appear to play a significant role in regulating *rsmA* in the wild-type PAO1 strain (Fig. 1B and C, 2, and 3). From this work, we conclude that AlgR and AlgU play a greater role in *rsmA* activation in *mucA* mutant strains. However, it is possible that *in vivo* conditions differ and that nonmucoid wild-type strains also have increased regulation of *rsmA* under other conditions.

The *mucA* mutation leads to AlgU and AlgR activation of *rsmA*, suggesting that RsmA may regulate specific targets in *mucA* mutant strains. RsmA is a posttranscriptional regulator that binds mRNAs, in many cases, at or near the ribosome-binding site of targets (54). It is likely that RsmA has unknown targets in a *mucA* mutant background due to transcriptional differences between nonmucoid and mucoid strains (55–59). Therefore, RsmA will bind only to targets that are present at a given time, supporting RsmA working as a rheostat. An alternative explanation is that our conditions of growth in the laboratory environment do not represent the conditions in the CF lung. While this is quite obvious, it is known that the mutation in *mucA* leads to alginate production *in vivo* (11, 60, 61). In addition, nonmucoid *P. aeruginosa* also has active AlgU and AlgR during infection because of alginate secretion by nonmucoid *P. aeruginosa* from CF patients (62, 63). Therefore, it is highly probable that AlgU and AlgR activity *in vivo* would increase RsmA levels during infection. What role RsmA plays due to AlgU and AlgR regulation *in vivo* is not known.

The significance of AlgU and AlgR control of *rsmA* is demonstrated by the analyses of RsmA targets. The posttranscriptional activity of the RsmA targets, *tssA1* and *hcnA*, was greater in the *mucA22* background than in the wild-type PAO1 strain (Fig. 7). We also observed a lack of posttranscriptional activity on RsmA targets when *algU* or *algR* was inactivated in a *mucA22* mutant strain (Fig. 7), due to the decreased expression of *rsmA*. Therefore, we conclude that RsmA is active in an *mucA22* mutant strain and that RsmA activity requires AlgU and AlgR to increase *rsmA* gene expression in this background. The correlation between increased AlgR activity and increased RsmA activity in *mucA* mutants also supports these systems acting as a rheostat to fine-tune gene expression, as opposed to an on/off switch. When AlgU and AlgR increase RsmA levels, the indirect effects of these two regulators may be due to RsmA. Further work is necessary to pursue this exciting discovery that could help explain the exclusive expression of particular virulence genes in a given background.

The role of AlgU and AlgR on the RsmA-antagonizing small RNAs *rsmY* and *rsmZ* is likely indirect. While our Northern blot data were not strong, they did support our transcriptional fusion analysis. Our transcriptional fusions mirrored what was seen in a previous study (28), supporting the idea of AlgR indirectly controlling *rsmY* and *rsmZ* expression. We hypothesize that AlgU and AlgR activities may coincide with factors that lead to increased GacA phosphorylation, resulting in the increased expression of *rsmY* and *rsmZ*. Further work is necessary to establish the mechanism for increased expression of the small RNAs in a *mucA* background.

Another question raised by our study is how RsmA remains active when the antagonizing small RNAs are increased. One possibility is that RsmA preferentially binds its targets in the *mucA* mutant strains better than the antagonizing small RNAs. This might also result from other regulators, either protein or other small RNAs that are currently unknown and affect the ability of RsmA to interact with the antagonizing small RNAs RsmY and RsmZ.

Altogether, our work demonstrates that AlgR is required for increased *rsmA* expression in *mucA* mutant strains. Phosphorylation of AlgR was not required for *rsmA* activation. The increased RsmA levels in a *mucA22* mutant strain result in increased RsmA activity, even though the antagonizing small RNAs are also increased. What roles RsmA plays and how RsmA functions in chronic infecting strains are not known. To better understand the role of RsmA, we are currently investigating new possible RsmA targets in AlgU-active strains. A further understanding of the RsmA regulon in *mucA* 

mutants may provide additional insight into how *P. aeruginosa* becomes such a successful CF pathogen and has implications for the important role of RsmA in all types of *P. aeruginosa* infections.

# **MATERIALS AND METHODS**

**Strains, plasmids, and media.** The strains used in this study are presented in Table 1. *Escherichia coli* strains were maintained on LB (Difco) plates or broth without or with antibiotics as appropriate. *Pseudomonas aeruginosa* strains were grown on *Pseudomonas* isolation agar (PIA), LB, or Vogel-Bonner minimal medium (64) and supplemented with the appropriate concentration of antibiotics. For *E. coli*, antibiotics were used at the following concentrations when appropriate:  $10 \mu g/ml$  tetracycline,  $15 \mu g/ml$  gentamicin,  $100 \mu g/ml$  ampicillin, and  $35 \mu g/ml$  gentamicin,  $50 \mu g/ml$  tetracycline, and  $300 \mu g/ml$  carbenicillin. For allelic exchange, sucrose was supplemented at 10% in YT (1% tryptone and 0.5% yeast extract) medium.

**Mutant construction.** All PCR products were amplified from *P. aeruginosa* PAO1, unless otherwise noted, using Q5 polymerase (New England BioLabs). Crossover PCR (SOE'ing) (65) was used to construct deletion mutations and to clone into the suicide vector pEX18Tc or pEX18Gm (66). All cloned constructs were confirmed via sequencing. *P. aeruginosa* strains were conjugated with *E. coli* as a donor strain and the pRK2013-containing helper strain (67). Conjugations were performed overnight on LB plates at 30°C, and conjugations were plated for single-crossover mutants on the appropriate selective media. Merodiploids were grown without selection and then screened for sucrose sensitivity on YT–10% sucrose plates. Mutations were confirmed using PCR with primers containing the suffix intF and intR shown in Table 1 and sequencing of the resulting PCR fragment. Hemagglutinin (HA) tagging of proteins was accomplished using primers containing the HA tag at the 3' end of the gene and introduced as described above using the suicide vector pEX18Gm.

**algR** mutant construction. The wild-type *algR* genomic region was amplified using Q5 (NEB) and primers algRXbalR and algRHindIIIF and cloned into pEX18Tc. Site-directed mutagenesis was performed using the algRD54XbalF/algRD54NR primers for the D54N allele or the algRD54Ef/algRD54ER primer pair for the D54E mutation. The *algZ* mutant was constructed using the algZHSDMF/algZHSDMR primers. The primers were phosphorylated and used in site-directed mutagenesis, in accordance with the manufacturer's instructions, using Q5 (NEB). Constructs were analyzed by restriction enzyme analysis and sequencing. Mutant strains were constructed using homologous recombination, as described above, and were checked using PCR and the algRintF/algRintR primer pair and digestion with the appropriate restriction enzyme. Additional PCR amplicons were sequenced to confirm the mutation in each strain using the same primers. Further confirmation of mutants was done using phenotypic assays.

Transcriptional and translational leader fusion analysis. Upstream DNA fragments containing promoter regions were generated by using primers listed in Table 1 in conjunction with Q5 polymerase (New England BioLabs). PAO1 genomic DNA was used as the template. PCR products were cloned into pMiniT (NEB) and then subcloned into miniCTX/acZ using the restriction enzymes HindIII and BamHI, HindIII and EcoRI, or KpnI and BamHI (NEB). The rsmA transcriptional fusions have been previously described (31). To construct rsmY and rsmZ transcriptional fusions, the rsmYTFFEcoRI/rsmYTFR and rsmZTFF/rsmZTFR primer pairs, respectively, were used. PCR products were purified, cut with restriction enzymes, and inserted into the EcoRI and BamHI sites of miniCTX/acZ using T4 DNA ligase (NEB). The leader fusion vector was constructed by annealing the lacUV5Notl/lacUV5BamHI primer pair together and cloning into the Notl/BamHI site of CTXCP (31). Translational/leader fusions were constructed using the tssA1annealF/R and hcnAannealF/R primer pairs (Table 1) and were cloned into the Scal/BamHI site of the leader fusion vector. Fusion constructs were confirmed by sequencing and conjugated into P. aeruginosa strains by triparental conjugation. Strains were selected for tetracycline resistance and then conjugated with pFLP2 to remove vector sequences (66). Strains were selected for carbenicillin resistance, grown overnight without selection, and plated on YT medium with 10% sucrose to select for the loss of pFLP2. Individual colonies were patch-plated onto VBMM CB300 and PIA to ensure the loss of pFLP2. To confirm the presence of the fusion constructs, PCR was performed using the forward primer used to construct the fusion and the reverse primer lacZRforTF (Table 1).  $\beta$ -Galactosidase activity was determined by incubating cell extracts with o-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml), as described by Miller (68). A strain carrying the empty vector miniCTX/acZ was also conjugated into PAO1 and assayed, and this background (28 Miller units) was subtracted from all transcriptional fusions. The translational/leader fusion backbone CTXCPlacUV5 had no background activity. All mucoid strains were confirmed mucoid at the end of each experiment by plating on PIA plates to ensure all colonies were mucoid. Three biological replicates were reproduced for all assays.

**AlgR purification.** The *algR* gene was PCR amplified using Q5 (NEB) and PAO1 chromosomal DNA using oligonucleotides algRSal1F and algRNot1R (Table 1). A 754-bp Sall/Not1 fragment was cloned into pGEX-4T-3 (Novagen) to be expressed as a glutathione *S*-transferase–AlgR (GST-AlgR) fusion protein. The resulting plasmid (pGEX-4TAlgR) was transformed into *E. coli* BL21(DE3) (NEB) cells and incubated overnight. The colonies from this transformation were collected, inoculated into LB supplemented with 100  $\mu$ g/ml ampicillin, and grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.6; 0.2 mM isopropyl- $\beta$ -D-galactopyranoside (IPTG) was added to induce AlgR expression for 4 h at 15°C. The cells were collected by centrifugation (6,740  $\times$  *g*, 15 min), washed once in 20 ml of phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS containing protease inhibitors (Thermo Fisher). AlgR was purified from this supernatant using the GST spin purification kit (Thermo Fisher). After binding of the fusion protein and

TABLE 1 Strains and plasmids used in this study

Str	ain or plasmid	Genotype or relevant properties <sup>a</sup>	Reference or source
E. (	coli strains		
I	NEB5 $\alpha$	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 φ80Δ(lacZ)M15 gyrA96	New England BioLabs
	SM10	thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km <sup>r</sup>	72
I	pRK2013	Helper strain	67
Pla	ismids		
	pEX18Tc	Allelic exchange vector	66
	pEX18Gm	Allelic exchange vector	66
	∆ <i>rsmA</i> pEX18Tc	Allelic exchange for <i>rsmA</i> nonpolar deletion	31
	∆ <i>algR</i> pEX18Tc	Allelic exchange for algR nonpolar deletion	47
i	algZHSDM/pEX18Gm	Allelic exchange for making <i>algZ</i> mutant	This study
l	rsmAHA/pEX18Gm	Allelic exchange for rsmA HA allele	31
	pGEX4T-3	GST fusion vector	GE Healthcare
	pGEX4T-3 algR	AlgR purification	This study
I		Transcriptional fusion vector	/3
I	rsmATF2/lacZ	Transcriptional fusion	31
	rsmATF3-lacz	Transcriptional fusion	31
	rsmaif2-lacz	Transcriptional fusion	31 This study
	ISINY IF I-IACZ	Transcriptional fusion	This study
	rsmVTE3_lac7	Transcriptional fusion	This study
	$rsm7TE1_{ac7}$		This study
	rsm7TE2-lac7	Transcriptional fusion	This study
	lacl IVSCTXCP	Leader fusion vector	This study
ĺ	laci IV5 tssA-lac7	tssA1 leader fusion	This study
Ĵ	laci IV5 hcnA-lac7	hcnA leader fusion	This study
Р.	aeruginosa strains		
	PAOT	Wild type	74
4	∆ <i>algK</i> mutant	alge mutant	75 This study
	D54N Mulani D54E mutant	algR with alutamate instead of aspartate at residue 54	This study
	ala7 mutant	ala7 mutant	This study
1	muca22 mutant	mucA22 mutant	
1	mucA22 AdlaR mutant	mucA22 Matant	This study
Ĵ	mucA22D54N mutant	mucA22 mutant strain with <i>alaB</i> with asparagine instead of	This study
		aspartate at residue 54	
	mucA22D54E mutant	mucA22 mutant strain with alaR with alutamate instead of	This study
		aspartate at residue 54	
	mucA22 algZ mutant	mucA22 mutant strain, algZ mutation	This study
	∆ <i>rsmA</i> mutant	rsmA nonpolar deletion	31
	mucA22 ΔrsmA mutant	rsmA nonpolar deletion in mucA22 strain	31
4	∆ <i>rsmY</i> mutant	rsmY nonpolar deletion mutant	This study
	∆ <i>rsmZ</i> mutant	rsmZ nonpolar deletion mutant	This study
I	PAO1 rsmATF1-lacZ	rsmA transcriptional fusion strain	31
	∆ <i>algR</i> TF1- <i>lacZ</i> mutant	rsmA transcriptional fusion strain	This study
	D54N rsmATF1- <i>lacZ</i> mutant	rsmA transcriptional fusion strain	This study
	D54E rsmATF1-lacZ mutant	rsmA transcriptional fusion strain	This study
1	mucA22D54N rsmATF1-lacZ mutant	rsmA transcriptional fusion strain	This study
1	mucA22D54E rsmATF1-lac2 mutant	rsmA transcriptional fusion strain	This study
1	mucA22 algZ mutant	rsmA transcriptional fusion strain	This study
	mucA22 IFI-lacz mutant	rsmA transcriptional fusion strain	31 This study
4	$mucA22 \Delta algk rsmATFT-lacz mutant$		
	PAUT ISMATES lacz mutant	rsmA transcriptional fusion strain	31 This study
1	$\Delta aigh IsinATF3-lacz mutant$	rsmA transcriptional fusion strain	21
	$mucA22 \Lambda JalaR rsm \Delta TE3-lac7 mutant$	rsmA transcriptional fusion strain	This study
1	PAO1 rsmATF2-lac7	rsmA transcriptional fusion strain	31
	$\Lambda a la R$ rsmATE2-lacZ mutant	rsmA transcriptional fusion strain	This study
	mucA22 rsmATF2-lacZ mutant	rsmA transcriptional fusion strain	31
	mucA22 $\Delta a la R$ rsmATF2-lacZ mutant	rsmA transcriptional fusion strain	This study
	PAO1 rsmYTF1- <i>lacZ</i> mutant	rsmY transcriptional fusion strain	This study
	∆ <i>algR</i> rsmYTF1- <i>lacZ</i> mutant	rsmY transcriptional fusion strain	This study
	algZ rsmYTF1-lacZ mutant	rsmY transcriptional fusion strain	This study
_ 1	mucA22 rsmYTF1-lacZ mutant	rsmY transcriptional fusion strain	This study

(Continued on next page)

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## TABLE 1 (Continued)

Strain or plasmid	Genotype or relevant properties <sup>a</sup>	Reference or source
<i>mucA22 ΔalgR</i> rsmYTF1- <i>lacZ</i> mutant	rsmY transcriptional fusion strain	This study
mucA22 algZ mutant	rsmY transcriptional fusion strain	This study
PAO1 rsmYTF2- <i>lacZ</i>	rsmY transcriptional fusion strain	This study
∆ <i>algR</i> rsmYTF2- <i>lacZ</i> mutant	rsmY transcriptional fusion strain	This study
mucA22 rsmYTF2-lacZ mutant	rsmY transcriptional fusion strain	This study
<i>mucA22 ΔalgR rsm</i> YTF2- <i>lacZ</i> mutant	rsmY transcriptional fusion strain	This study
PAO1 rsmYTF3- <i>lacZ</i>	rsmY transcriptional fusion strain	This study
mucA22 rsmYTF3-lacZ mutant	rsmY transcriptional fusion strain	This study
PAO1 rsmZTF1-lacZ	rsmZ transcriptional fusion strain	This study
∆ <i>algR</i> rsmZTF1- <i>lacZ</i> mutant	rsmZ transcriptional fusion strain	This study
algZ rsmZTF1-lacZ mutant	rsmZ transcriptional fusion strain	This study
mucA22 mutant	rsmZ transcriptional fusion strain	This study
<i>mucA22 ΔalgR</i> rsmZTF1- <i>lacZ</i> mutant	rsmZ transcriptional fusion strain	This study
<i>mucA22 algZ</i> rsmZTF1- <i>lacZ</i> mutant	rsmZ transcriptional fusion strain	This study
PAO1 rsmZTF2-lacZ	rsmZ transcriptional fusion strain	This study
mucA22 rsmZTF2-lacZ mutant	rsmZ transcriptional fusion strain	This study
PAO1HA	PAO1 with epitope-tagged RsmA	31
∆ <i>algR</i> HA mutant	$\Delta algR$ mutant with epitope-tagged RsmA	This study
mucA22HA mutant	mucA22 mutant with epitope-tagged RsmA	31
<i>mucA22 ΔalgR</i> HA mutant	mucA22 $\Delta algR$ mutant with epitope-tagged RsmA	This study
PAO1 lacUV5 hcnA-lacZ mutant	PAO1 with <i>hcnA</i> leader fusion	This study
mucA22 lacUV5 hcnA-lacZ	mucA22 mutant with hcnA leader fusion	This study
<i>mucA22 ΔrsmA lacUV5 hcnA-lacZ</i> mutant	mucA22 $\Delta rsmA$ mutant with hcnA leader fusion	This study
<i>mucA22 ΔalgU lacUV5 hcnA-lacZ</i> mutant	<i>mucA22</i> $\Delta algU$ mutant with <i>hcnA</i> leader fusion	This study
<i>mucA22 ΔalgR lacUV5 hcnA-lacZ</i> mutant	<i>mucA22</i> $\Delta algR$ mutant with <i>hcnA</i> leader fusion	This study
PAO1 lacUV5 tssA1-lacZ	PAO1 with <i>tssA1</i> leader fusion	This study
mucA22 lacUV5 tssA1-lacZ mutant	mucA22 mutant with tssA1 leader fusion	This study
<i>mucA22 ΔrsmA lacUV5 tssA1-lacZ</i> mutant	mucA22 $\Delta rsmA$ mutant with tssA1 leader fusion	This study
<i>mucA22 ΔalgU lacUV5 tssA1-lacZ</i> mutant	mucA22 $\Delta algU$ mutant with tssA1 leader fusion	This study
<i>mucA22 ΔalgR lacUV5 hcnA-lacZ</i> mutant	mucA22 $\Delta algR$ mutant with <i>tssA1</i> leader fusion	This study

<sup>a</sup>Km<sup>r</sup>, kanamycin resistance.

washing the column, AlgR was cleaved away from GST with 10 U of thrombin/column overnight at 22°C. Purified protein was dialyzed using a Slide-A-Lyzer (Thermo Fisher) and storage buffer (20% glycerol, 20 mM Tris [pH 7.5], 5 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol [DTT]) overnight at 22°C. The identity of the purified protein was determined by mass spectrometry. The purity of AlgR was visually determined in a Coomassie-stained 7.5% electrophoresis gel (SDS-PAGE).

**EMSAs.** Purified AlgR was used in electrophoretic mobility shift assays (EMSAs) using either PCR amplicons or annealed primers using the LightShift kit, in accordance with the manufacturer's instructions (Thermo Fisher Scientific). For *rsmA*, the rsmAPE3F/rsmAPE3R, rsmAPE1F/rsmAEcoRIR, or rsmAPE3F/rsmAEcoRIR primer pair was used to produce PCR amplicons using *Taq* polymerase (NEB). Amplicons were gel extracted and biotinylated using the 3' biotinylation kit (Thermo Fisher Scientific). Annealed primers were heated to 95°C for 5 min and cooled to room temperature. The annealed primers were biotinylated and incubated with purified AlgR. Purified AlgR was incubated at increasing concentrations to determine the suitable concentrations to be used. The nonspecific competitor poly(dl-dC) at 25 ng/ $\mu$ l was used for all gel shift reactions. DNA and protein were electrophoresed through 5% or 10% native polyacrylamide gels, transferred to a nylon membrane using a semidry apparatus (Hoefer), UV cross-linked, developed using the chemiluminescence detection kit (Thermo Fisher Scientific), and visualized using a charge-coupled-device (CCD) camera (ProteinSimple). The gel shift assays were repeated at least two times.

Western blot analysis. *P. aeruginosa* strains were grown in LB at 37°C for 8 h. The bacteria were collected by centrifugation, resuspended in sterile PBS, and lysed by sonication using a Branson sonifier. Total protein concentrations were determined by the Bradford protein assay (Bio-Rad, Carlsbad, CA). Cell extracts (5 to 10  $\mu$ g) were separated by 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and probed using a 1:2,500 dilution of anti-HA monoclonal antibody in blocking buffer (Thermo Fisher, Pittsburgh, PA), followed by a 1:30,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (69). Densitometry of Coomassie-stained SDS-PAGE gels was used to standardize the Western blots using total protein analysis, as described previously (70, 71). Detection was performed using the ECL Plus kit (Thermo Fisher) and chemilumines-cence detection (ProteinSimple, Santa Clara, CA). The blots and Coomassie-stained SDS-PAGE were analyzed using ImageJ analysis. All Western blotting was repeated at least four times.

**Primer extension.** Total RNA (15  $\mu$ g) from *P. aeruginosa* was isolated using the PureLink RNA minikit (Life Technologies, Grand Island, NY). Radiolabeled primers rsmYPE1 and rsmZPE1 (see Table 1) were used in a reverse transcription reaction using ThermoScript (Life Technologies). A temperature of 42°C was used for the extension. The same primer used for primer extension was also used in a sequencing reaction with the *rsmY* or *rsmZ* upstream sequence in pGEM-T (Promega, Madison, WI) using the

# TABLE 2 Primers used in this study

algfinthfCCAACTGCACTGCCAGGTGCMutantalgfinthfCCGCACTGCTCATCGCACAGGCAGMutantalgfinthfCCGCGCGCGCACGCACGCACAGGCACCGCACAGGCACGGCAGMutantalgZbindfillFCCGCGACGCTCCTGCTCACGCACCGCCACAGCACAGCAC	Primer name	Sequence	Use <sup>a</sup>
alg8inHRCCCCCATCGTCACCGCCACAGACAGAACGGMutantalg7binHIRCCCCCATCGTCACCACCACCAGCACCAGCAGCAGCAGCAGCAGCAGCAGC	algRintF	GCAACTGGACTGGCAGGTGC	Mutant
ajdBamHill CCCCCCACAACCAACAACAACAACAACAACAACAACAAC	algRintR	CGCGACTGGTCATCGGCAG	Mutant
alg2HindlifferGCGCAAGCTTCTCGCTCGCACAAGAAACGGMutantalgRD5MRbckFrCACGTGGCGGCGAGAACTGCACMutantalgRD5MRCACGAGGCGGCGGAAGATCGGCGCTGACMutantalgRD5MRGCATCTCTAAGAGTCGCGCTGAGMutantalgRD5MFGTATCTCGGCTGCGCGTGAGMutantalgRD5MFGATATCCGGCTGCGCGTGAGMutantalgRD5MRCGGGCGAATGCGCGCTGCGMutantalgRD5MRCGGGCGAATGCGCCGCTGAGAGCMutantalgRD5MRCGGGCGAATGCGCCGCTGAGAGCAGGGGGGGGGGGGAlgR pufficationalgRSNIFCCGGCCGACTGAAGAGCCGGTGCGGGGGGGGGGGGGGGG	algRBamHIR	GCGCGGATCCGTCAGAGCTGATGCATCAGACG	Mutant
algZDSMcheckFCACCTGGGCGGAGAACTGACMutantalgRDS4XBaFAACATCGCATGACCGGTGAGACGGCMutantalgRDS4RFGTCTTCTAGAGATCCGCATGACMutantalgRDS4RFGTCTTCTAGAGATCCGCATGCMutantalgRDS4RFGATATCGGGCTTGAGAGCTGTGCMutantalgRDS4RFGATATCGGGCTGAGAGCTGTGCMutantalgRDS4RFGGGCCAACTGAGACGTCGTCGAGACGAMutantalgRDS4RFGGGCCTAGAGCGCCGCTGCAGAGCGCGCGCGCGCAGAGAGCGCGCGC	algZHindIIIF	GCGCAAGCTTCTCTCGCTGCAACAAGAAACGG	Mutant
algRD54KbalfALCATCGCGCTGCGCGCGCGCGCGCMutantalgRD54FFGTCCTTCTAGAGATCCGCGCTGAMutantalgRD54FFGTATCTCGGCGCTGAGGCTGAGMutantalgRD54FFGATATCCGCCTGCGCTGAGMutantalgRD54FFGATATCCGCCTGCGCTGAGGMutantalgRD54FFCGGGCGAATCGCGCCCTGAGAGCAGAMutantalgRD54FFCGGGCGAATCGCGCCCTGAGAGCAGAGGGGAGAGGGGGGGG	algZSDMcheckF	CAGCTGGGCGGAGAACTGAC	Mutant
algRD54RRCACCAGCACCATTCCGGCCTGAMutantalgRD54FFGTCTTCTAGAGATCCCGTGACGATGCMutantalgRD54FRGATATCGGCCTGACGACGTGCGMutantalgRD54FRGATATCGGCCTGACGACGCCTGCGMutantalgRD54FRGGATCTCTGTTCACAGCCGCCTGCAGAGACGACGACGACGACGACGACGACGACGACGACGA	algRD54XbalF	AACATCCGCATGCCCGGTCTAGACGGC	Mutant
algB054EF GTCTTCTA6A6ATCCGCATGC Mutant algD55EF GATCCGGCCTTGCGACGCTGCG Mutant algD55ENMF GATCCGGCTGCGACGCGCGCGCGACGACGGCGGACGACGGACG	algRD54NR	CAGCAGGACGATATCGGGCTTGA	Mutant
algRb3E4R GATATCGGGCTTGAGGCTGTGA algZH5DMR GATATCGGGCTTGAGGCCTGACAG algZH5DMR GATATCGGGCTGACAGGCTGACAGAG algRball CCGGCGCAGCCGGCCTGAGAGGCTGATGACAGAG AlgRball CCGGCGCGCCGCCGCAGAGCTGATGCATCAGAGG AlgRball CCGGCAGCGGCGCTCAGAGCTGATGCATCAGAGG Mutant rsmARbbal GCGCTTAGAGCACCGGTGAGGGGGGGGGGGGGGGGGGGG	algRD54EF	GTCCTTCTAGAGATCCGCATGC	Mutant
alg2HSDMF GAATTCCTGTTCAACAGCCTGAACAG Mutant algRSalF CGCGCCGACCAACTCGCGCCTGCA AlgR purification smARHindll CGCGCAACTCGGCGCCTGACAGCTGACGCCTGAC Mutant smARHindll GGCGAACTCGGGCCAACTCGCGCCGCGGG Northen blot smARHindll GGCGAAGCTGGGCCAACTCGCGCGGGG Northen blot smXPRAF GTGGGCGCCACGGTGATCCTGGCGGG Northen blot smXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC Northen blot smXPRAF GGGCCCGCCTGGGGACCGGGGGGGTATTAC Northen blot smXPRAF GGGCCCCACTCGTGGGGACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGGACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCCACTCGTGGGAACAC SmXPRAF GGGCCCACTCCTGGGAACAC SmXPRAF GGGCGCCACCCTCGTGGACAC SmXPRAF GGGCGCCACTCCTGGGAACGCGACAC SmXPRAF GGGCGCACACTCCTGGGAACGCGACAC SmXPRAF GGGCGCCACCCTGCTGGGAACGCC SmXPRAF GGGCGCACACCCTCTGGGAACGCCACGCGGGGGGTATTAC Northen blot SmXPRAF GGGCGCAATTCACTGGGAAGCCGACGCG SmXPRAF GGGCGCGAATTCACTGGGAAGCCGACGCG SmXPRAF GGGCGGAATTCACTGGGAAGCCGACGCG SmXPRAF GGGCGGAATTCACTGGGAAGCGCGGGGG SMXPR SmXPRAF GGGCGCGACCGGCGGGGGGGGGG SMXPR SmXPRAF GGGCGCGACCGGCGGGGGGGGGG SMXPR SmXPRAF GGGCGCGCCCACCGGCGGCGGGGGGGGG SmXPR SmXPR SmZPF GGTTACTGGCAAGTGGGGGGGGGGGGGGG SMXPR SmX	algRD54ER	GATATCGGGCTTGAGGCTGTCG	Mutant
alg2HSDMR CGGGCGAATCCGCGCCTGCA algRball CCGGCGCGCCGCGCGCGAGTGCGATGCGATGCATGCAGCAG AlgR purification algRball GCGCTCTGAGCGCGCGGTGCTGCGAGTGCATGCGAGCA Mutant rsmARbbal GCGCTCTGAGCGCGCGGGTGCTCGCGCGAGCA STMARbbal GCGCTCTGAGGCACGGGAGCGCGGG Mutant rsmARbbAlcekk GCCAAGGTTCGCTGGGCAGGCGAGGA STMARbAC rsmYTRPAFA CTGGAGCGAGGGCGGGGGACTGCGCAGGAG Northern blot rsmZRPAFA GGGCCCCATCGCGGCAGGGAGG SGGCGGGGGGGTGTGCGGCGGGGGGGGTATGC Northern blot rsmZRPAFA GGGCCCCATCGTGGTGTGGCGGCGGGGGGGGGGGGGGG	algZHSDMF	GAATTCCTGTTCAACAGCCTGAACAG	Mutant
algRSallF CCGGCCGACCGGCGCGCACCGCGGGGGCGGCGGCGGGGGG	algZHSDMR	CGGGCGAATCCGCGCCTGCA	Mutant
algNotlR AGCCCGGCCCCCACGAGTCGAGCGGAGCCGGAGC smARbbd GCGCTCTAGAGCACCGGGAGCCGCGAGCA smASbbd/heek/F GCCAAGGTTCGTCGGCAACATCACCACGCGGG SmSASbbd/heek/F GCCAAGGTTCGTCGGCAAGAACATCACCACGCGGG SmSASbbd/heek/F GCCAAGGTCCGTGGGGAGGAG SmSYTRPAFA CATGCGACGTCAGGACATTGCGCAGGAAG SmSYTRPAFA CATGCGCACGCAGGAGA SmSYTRPAFA GGGCCCCATGCGGGCAGGAGA SmSYTRPAFA GGGCCCCATGCGGGCAGGAGA SmSYTRPAFA GGGCCCCATGCGGGCAGGAGA SmSYTRPAFA GGGCCCCATGCGGGCAGGAGA SmSYTRPAFA GGGCCCCATGCGGGCAGGAGA SmSYTRPAFA GGGCCCCATGCGGGCGGGGGGGGGGGGGGGGGGGGGGG	algRSallF	CCTGTCGACATGAATGTCCTGATTGTCGATGAC	AlgR purification
rsmARkbal GCGCTCTAGAGCACGGTGATCCTGCAGACC Mutant rsmARkbal GCGAAGCTTGCGCAACATCACCACCCGCGGG Mutant rsmASDMkheckF GCCAAGCTTGCGCAACATCACCACCCGCGGG Northem blot rsmYRPAF GCTGGAGCACTTGCGCAGGAGA Northem blot rsmYRPAF GGCGCGCCCCACCCGTCATAGGGAGACATCACCACCGCGCGGGGTATTAC Northem blot rsmZPARAFT GGCGCGGATCCTCTGCGGTCGGGCCGGGCGGGGGTATAC Northem blot rsmYRPAF GCGCGGATCCTGCGTGCGGTCGGGCCGGGGGGGTATAC Northem blot rsmYRPAF GCGCGGATCCTGCGGTCGGGCCGGCAGGGGGGGGTATAC Northem blot rsmYTFRBamHI GCGCGGATCTCTACTGGGCAGAGAGC TF rsmYtFRBamHI GCGCGGATCCTGCGGCCGGACGGGGGGGGGGGGGGGGGG	algRNotIR	AGCCGCGGCCGCTCAGAGCTGATGCATCAGACG	AlgR purification
rsmAFIIndIII GCGCAACGTTCGGCAACATCACCACGGGG smVRPAF GCCAAGGTTCGTCGGCAACATCACCACGGGG rsmVRPAF CTGGACGTCAGGACATTCGCGCGGGAGA rsmVRPAF TATCGACTGCACTGACGACATTAGGAGACACACACACACA	rsmARXbal	GCGCTCTAGAGCACGGTGATCCTGCAGACC	Mutant
rsmASDMcheckF GCCAAGG1TICCAICGICGG PCCAGGAACATCGCCGCAGGAAG Northern blot rsmYRPAF CIGSACGTCAGCACATGCGCCGCGCGGGGGAACAC Northern blot rsmZPAF GGCCCCATCCTGCGGACGGAACAC Northern blot rsmZPAF GGCCCCATCCTGCGGTCAGGGACAACAC Northern blot rsmZPAFT GGCCCCATCCTGGCGACGGAACAC Northern blot rsmZPART7 TATACGACTCACTATAGGGAGAACAC NORTHALAGAGGGGGGGGGTATTAC Northern blot rsmZPART7 GGCGCGAATCGGTAGGCGTGGGCCGGCGGC rsmTFRBamHI GCGCGGATCCGTCGTGCGGCGGGGGTATTAC TF rsmYTFRBamHI GCGCGGAATCCCATGCTGGGGAGGGGGGGGGGGGGGGGG	rsmAFHindIII	GCGCAAGCTTCGGCAACATCACCACGCTGGG	Mutant
rsmYTRPAF CTIGGACGICAGGACATTAGGGAGAGAG Northern blot rsmZtPAFA GGGCCCCATCCTGCGTACAGGAGACATAAAAAAACCCCCGCCTTTGGG Northern blot rsmZtPAFA GGGCCCCATCCTGCGTACAGGGAACAC Northern blot rsmZtPAFT TAAACGACTCACTCATAGGGAGACACA SAAAAAGGGGCGGGGGTATTAC Northern blot rsmZtPAFT GGGCCGGAATTCCATAGGGGAGACAC TF rsmTrFH268mHH GCGCGGGATTCCATAGGGCAGCACAAAAAAAAAGGGGCGGGGGTATTAC TF rsmTrFH268mHH GCGCGGGATCCCCTCACGCGTGGTGGGCGGC TF rsmTrFH268mHH GCGCGGGATCCCGTCGCGCGGTGGTTTGGCTG TF rsmTrFH0AS GCGCGGATCCCATCGTGGGAGAGGGCGG Mutant rsmZXbalR GCGCGGATCCCGTCGGCGGAGAGGGCG Mutant rsmZXbalR GCGCGGATCCCGTCGGCGAGACGTCC Mutant rsmZxbalR GGCGCGAATCCGGTGGGGGATTGCCGGCGAACACC Mutant rsmZxbalR GCGCGGACTCCCGTCGGCGAACGTCCGCAACATCC Mutant rsmZxbalR GGCGCGGACCCCGTCGGCGAACGTCCGCAACATCC Mutant rsmZxbalR GCGCGGCTCAGAGAGGCGAACCCGGCGAACATCC Mutant rsmZxbalR GGCGGCTCAGAGAGGGCAACCCGCGCGAACATCC Mutant rsmZxbalR GGCGGCTCAGAGAGGGCACCCGCCGACCATCC Mutant rsmZxbalR GGCGGCTCAGAGAGGGCAGCCCGCGCGAGACACCCGC Mutant rsmXVDR CGCGGGCGAGCCCGCTGCGGAGACCCGCCGCGAGGAAACCCGCGGC Mutant rsmYXOR GGCGGCTGAACGGGGGGGGGCG Mutant rsmYXOR GGCGGCTGAACGGCGGGAGGCCGCGAGACCCGCGCGGG Mutant rsmYXDR GGAACTCGCGAGGTGCCCGAGGCTGCCGGGGGAAACCCGCGGGG Mutant rsmYNDR GGCGGCTTACACTTGAGGCGGGGGGGCGGGGGGGGGGG Mutant rsmYNDR GGCGGCTTACACTTGAGGCGGGGGGGGGGGGGGGGGGGG	rsmASDMcheckF	GCCAAGGTTTCCATCGTCGG	PCR check
rsmY1/RPAR IAALAGACICACIAIASGAGACAAIAAAAAACCCCGCUITIGGG Northern blot rsmZRPAF GGCCCCATCCTGCGGGACAGACAC Northern blot rsmZRPART7 TATATCGACTCACTATAGGAGAACAC Northern blot rsmZRPART7 TATATCGGACTCACTATAGGGAGACAC Northern blot rsmZRPART7 TATATCGACTCACTATAGGGAGACAC F rsmYTFFEBamHI GCCGGGAATCCTCCTGCGCCGAGTGCCGGC F rsmYTFFRBamHI GCCGCGGATCCTGCTGCGGCGGAGGCTGACG rsmSthonEcoRF GCCGGGACCGCGCAGGGGTAGACGCAGAGGCTGAC rsmZRpAIF GCCGGGACCCCTCTGCGGCGGGGAGCGCG Mutant rsmZXpalF GCGCGGATCCCCTGTGCGGCGGGAGGCGGA rsmZRPART GCCGGGACGCCCCACGCTGTTGGCGG Mutant rsmZXpalF GCGCGGATCCCCCTGTGGGGAGGCGCG Mutant rsmZXpalF GCGCGGACCCGTCTGGGGGAGGCGCG Mutant rsmZXpalF GCGCGGGACCCCGTCGGGGCGGCGCGCG Mutant rsmZXpalF GCGCGGGACCCCGCGCCGCGCGGGGGGGGGGG Mutant rsmZXpalF GCGCGGGACCCCGCCGCGGCGGCGCACCCCGC Mutant rsmZXpalF GCGCGGGACGCCGCGCGCGCGCGCGCGC Mutant rsmXXOF GCGGGAGCTCTGTGCGGGATTTGCGGGACTTGCGGGACTG Mutant rsmXXOF GCGGGAGCTCCTGTCACCGGCGCCGCGCGCGCGCGC Mutant rsmXXOF GCGGGAGCTCGCGCGCGCCGCGCGGGGCGGGGGGGAAACCGCCGGGGGG	rsmYRPAF	CTTGGACGTCAGGACATTGCGCAGGAAG	Northern blot
STM2HPAFGGGCLCCACITCL/GGGIACAGGGAAAAANorthern blotSTM2RPART7TAATACGAATCACITATAGGGAAGAAAAAAAAGGGGCGGGGATATACNorthern blotrsmYTFREARGCGCGGAATTCCTCTGCGCACCGGCCGGCGGCGGGGACAGAAAAAAAGGGGCGGGGGTATTACTFrsmYTFREARSGCGCGGAATTCCTCTCGCGCACGGGCGGGGCTFrsmYTFREARSGCGCGGACCCCATCCTGCGGAAGGCGGGGTFrsmYTFREARSGCGCGGATCCCCATCTGGGAAGGCGGGGTFrsmYTFREARSGCGCGGATCCCCATCGTGGGAAGGCGCGWutantrsmZxbalRGCGCGGTACCCGGTGGGGGGGGTTTGCCTGCCGTTTACTCGTCMutantrsmZSDEFGTTTTCTGGCAGGGAGGGGGGGGGGGGGGGGGGGGGGGG	rsmYT7RPAR	TAATACGACTCACTATAGGGAGACAATAAAAAACCCCGCCTTTTGGG	Northern blot
rsm2HPARI/IAAIACGACICACIALGAAAAAAAAAGGGCGGGGGIAIACNorthern blotrsm3TFFECoRIGCGCGAATCTGTGTGCCGGCGGCGCGGCTFrsm3TFFEARGCGCGGATCCTTCCTGCGCAAGTGTCTGACGTFrsm3TFFUASGCGCGGGACCCCCAGCGTGTAAGAGCAAGGCTTACTFrsm3thoftCoRIFGCGCGGAATCCAAACGGCGGGGGTGGGTCGGCMutantrsm2XpnIFGCGCGGTACCCCATGCTGGGCAAGGGCGMutantrsm2XpaIRGCGCGGTTGCGCGAGGTGTGTGGCGGAAGGGCGMutantrsm2XpaIRGCGCGCTTGGGCGAGGAAGCGCGAAACCGCCGCGTTTACTCGCMutantrsm2XpaIRGCGCGCTGCGGCGCGCACCGGATCCGCGCAGAAGGCGGMutantrsm2XpaIRGCGCGCGCGCGCCGCAGCGGATCCGCGCAGAGGGMutantrsm2XpAIRGCGCGCGGCGCGCCACCGGCGCGCGCGGCGGGMutantrsm2XpTRRCGGGGAGGCGCCACCGGCGCGCGCGGCGGCGGGGGGGGG	rsmZRPAF	GGGCCCCACTCCTGCGTACAGGGAACAC	Northern blot
TSmT1HECONICLGCGAATICCIGGICGGCCGGCCIPTSmTSTRBamHiGCGCGGATCCTTCTCTGCGCAAGATGTCCTGACGTFTSmTSTRBAmHiGCGCGGATCCTTCTCTGCGCAAGAGGCTTACCTFTSmSyshortEcoRiFGCGCGGATCCCATIGCTGGGAAGGGCCGMutantTSmZSbalRGCGCGGATCCCCATIGCTGGGAAGGGCCGCCAACATCCMutantrsmZSbalRGCGCCGTACCCGCCACCGCCCACCGCCCACCTCCMutantrsmZSDEFGTTTCTGGCAGGTGCGGGAATTCCCCGCCAACTCCCCGCCAACATCCMutantrsmZSDEFGTTTCTGGCAGGTGCGGCAGCCGCCACCTGCCGAACCTGCCGAAACCMutantrsmZSDRGCGCGCGCCCCCCCCCGTCCGACCGCCGCCGCAACCTGCCGCGAACCTGCCGCGAACCTGCCGCAACCTGCCGACGTGMutantrsmZNtRCATGCAGGAATTCATCGAGCTGMutantrsmYKORCGCGGACGTCCGTGTTCACTCGGCGAGCCGACGCGCCGAGGAAAACCGCGGTGMutantrsmYSOFFGCGTAAATCTTCAAACCGTCAGGACTCCAGGCCCGAGGAAAACCGCGTGGMutantrsmYSOFRCGGCGACGTCGGAGGGCCCCGAGGACTCCAGGCCCGAGGAAAACCGCGTGGMutantrsmYintFCCTGGAAGTGCGCCGCAGGATCCTGACGGCTGGAAGAACCGCGGTGGMutantrsmYintFGGCGGCTTAGATTTCCCGGCCGCGGAGACTCCAGCGGTGGAAAACCGGGTGGMutantrsmYintFCCTGGACGTGACGGGCCMutantrsmYintRGGCCGCTTTACACTTTACGTGCGCCGCGAGAGCTGAAGCGGGGGLeader fusion vectorlacUVSbamHIGATCCTCCACACATTAACGAGACGGCGCGGCGGCGGAAGCCGCGAGGACTGAAACGCLeader fusionrsmYintRGGCACGGGAGCGTTGAAGGGCGAGGGGGGGGGGGAGAGAGGGCGGGGGGGG	rsmZRPART/		Northern blot
TSmT1HBamHICLGCGGATCCTTCCLGGCAAAGTGCCGAGGIFrsmTFFD4SGCGCGCGCCCCCCAGCGTGAGTAAGCCAAGGCTACTFrsmYshontCoRIFGCGCGGATCCCATGCTGGGCAAGAGGCGCGMutantrsmZkpnIFGCGCGGATCCCCATGCTGGGCGAAGGGCGCGMutantrsmZkbalRGCGCTTAGAGGGGATCCCGCCAGCGTGGTTTACTCGTCMutantrsmZSDEFGTTTCTGGCAGGAAGCCGCCAACATCGCCGAAAACGMutantrsmZSDEFGTTTCTGGCAGGCGCGCGCGGCGGCGGCGGCGMutantrsmZNDTFGCGCGCGGCGCCGCGCGGCGGCGGCGGCGGGGGGGGGG	rsmYTFFEcoRI		
SmiThPUSGCGCGGATCCCAGCGTGTAGCCGGIFsmiTshottEcoRIFGCCCGGATCCCCAGCGGTGGTTGCGGCGTGGTTFsmZKpnIFGCGCGGATCCCCGTCTGCGGGAGAGGGCGMutantsmZKpnIFGCGCGGTCTGGGGCGCGAGAAGGGCGMutantsmZXbalRGCGCTTAGAGAGGCGAAATCCGCCAACTCCMutantsmZSOEFGTTTTCTGCGAGGTTGCGGGCAATTCGCGCGCAACCTGCCAGAAACCMutantrsmZSOEFGCGCGCGCCCCCGACCGGCCGCAACCTGCCGCACCTGCCAGAAACCMutantrsmZsotFGCGCGCGCCCCCGACCGTCCGTGCGGAGCTGMutantrsmZintFGCGCGCGCCCCCCGACCGTCCGTGCCGCACCTGCCGAGACTGCMutantrsmYK0FCGCGGAGCTCTGGCAGCCCGCACCGCGCCGCGCGCGCGGCGGMutantrsmYS0EFGCGTAAATCTCGAAGCACTCCGCCCGAGGGAAACCCGCGCGCMutantrsmYS0FCGCGGAGCTGGCCGCAGGGGGGGGGCGCGCGGGGGGGGGG	rsmYTFRBamHI	GCGCGGAICCIICCIGCGCAAIGICCIGACG	
TSmTSIDItECoNIPGCGCGAATICAAAGGGCGIGGAAGGCTCGCIFrsmZxbnlFGCGCGGATCCCCATGGCGGAAGGCTCGCMutantrsmZxbalRGCGCGGTCCCCAGGGAAGCGCGAACATCCMutantrsmZxbalRGCGCGCTCGGGGAAGCGCGGAACATCCMutantrsmZxbalRGACGAGTAAAACGGCAGGCAACATCCMutantrsmZxblRGACGAGTAAAACGGCAGGCAACATCCCGCCAGAAAACMutantrsmZsDERGACGAGTAAAACGGCAGCGCGCGCGCCGCCGCCGCCGAGAAAACMutantrsmZintFGCGCGCGCGCCGCCGCCGCCGCCGCGCGCGCGMutantrsmYNFRCATGCAGGAATTCATCGAAGCCCTGCGCGCGCGCGGCGGAGAAACCGCGTGMutantrsmYSOEFGGGTAATCTTCAACGGATCTCGGCGCCGCAGGATCTGGGCGCGGAGAAACCGCGCTGMutantrsmYSOEFGGGTAATCTTCAACGGCTCGCAGGCCCGCAGGATCCTGAGGATTGAGAGATTACGCMutantrsmYsOEFGGAATTCAGGAAGGGGTGCCCMutantrsmYintFCCTGGAGCTGAAGGTGTCCCMutantrsmYintRGGACGCTTACACTTTATGCTTCCGGCCGCAGAGCCGCGAAGGATCAAGGCLeader fusion vectortsA1annealFGATCCTCCCACACTTATAGGAGGGAAGCGTGAAGCCGGCGCAAGCATAAACGCLeader fusiontsA1annealFGTCCATCTCCTCTCCGGGCGGAGAAGCGGCGCGCGCGCGC	rsmyTFFUAS		
dnf1rhsdnfmlGCGGGTACCCGGTCGGGGAAGGCCGMutantrsmZxbalRGCGCGTACCCGGTCGGCGCAGAAGGCCGMutantrsmZxbalRGCGCGTACCCGGTCGGGGATTGGCGGCGCCAGAACCGCCCACCTGCCAGAAACCMutantrsmZSDEFGTTTTCTGGCAGGTTGCGGGATTGGCGGCAGAACCGCCCAGCAGACCMutantrsmZntFGCGCGGTCACAACGCCGACCGATCCGTGCGAGCTGMutantrsmZNtFCGCGGAGCTCCTGTCACGCGCGCCGCAGAGCGMutantrsmZntFCGCGGAGCTCCTGTCACCGGACGCGCCGCAGGAGGCGMutantrsmYKOFCGCGGAGCTCCTGTCACCGCGCGCCGCAGGAGGCGMutantrsmYSOERCGCGTCTAGATTCGCCGACGCGCGCGCGAGGAACTCCGGCGCGAGGAAAACCGCGTCGMutantrsmYSOERCGCGGAGCTCCTGGGCCCGAGGAGCCCGAGGATACCGCGGCGMutantrsmYNTFCGAGCGGGGGGGCGCCGCGGGGGCGCCGAGGATCCTGACGGTTGAAGATTACGCMutantrsmYNTRGGAATTCAGGAAGGTGCCCMutantlacUVSNotlGCCCCTTTACACCTTATACGGACGGAGGCALeader fusion vectorlacUVSNamHIGATCCTCCACACATTATACGAGCGGAAGGCAATAGCCLeader fusiontsx1annealFGATCCTCCTCTCCTGCGGGCGAGGAAGGCAATGCACLeader fusionhcnAannealFGGCATTGCCCTTTCATCCTGTGAGGAGGCAATGCACLeader fusionhcnAannealRGTGCATTGCCCTTTCATCGTGAGGAGAGGGGNorthern blotlacZScalFGATGGACTGCACCTATAGGGAGGAGGCGGCGGCGGCGGCGGCGGCGGCGGel shiftlacZRholCCCCCTGGAGCAGACATTGCTTCCGGAGGAACTTCGCGCGCG			
SIMEAphineGCGCCTAGAGAGCGAAACGGCGAAACGGCGMutantrsmZSDBRGCGCTCTAGAGAGCGAAACCGCCAACATCCMutantrsmZSDEFGTITTCTGGCAGGTTGCGGAAATCCCCGCAACCTGCCMutantrsmZSDERGACGAGTAAAACGGCAAATCCCGCGCACCGTCCCGCAAAACCMutantrsmZintFGCGCGCCGCCCCGCACCGTCGCGAACCTGCMutantrsmZNDRCATGCAGGAATTCATCGAGCTGMutantrsmYKOFCGCGGACCTCTGTTCACTCGAAGCACTCCAGMutantrsmYKOFCGCGTAAGTTCATCGCAACTCCGCAAGCACGCGCGGGCCGAGGAAACCGCGCGMutantrsmYSDEFGCGTATCTTCAAACCGTCAGGATCCTGCGGCCCGAGGAAACCGCGCGGMutantrsmYSDERCGACGCGGTTTCCTCGGGCCGCAGGATCCTGACGGTTGAAGATTACGCMutantrsmYinfFCCTGGAACTGGACGGGAGMutantrsmYinRGGACTCTCCAACATTATACGCACCGGCCGGAAGCATTAAGCLeader fusion vectorlacUVSNotlGGCCGTGACGTGACCGTAAGAGGCGCAGCGAAGCATAAACTGTAAAGCLeader fusiontsx1annealFGATCCTCCGATGATAGGGAGGACTGCCCCGAAGGATGGCGCLeader fusioncrsnAnnealFGATCCTCCCATCATCATCGGAGGGCAATGCACLeader fusionhcnAannealFGATCCCCTCTCTCTCCCCAGAGAGGCAATGCACLeader fusionfsrRNAITTGGACGGAGGGGGGGAGNorthern blotSrRNAIGCGCTGCGCAGCCCGCCGGTTATAGTF vectorlacZRAbolCCCCCTGGAGAGAGGGGGAGGAGAGAGAGGGCGATGGGSEQrsmARSDMAIgRFGAATGCGCGAGCGGGGGGAGGAGAAAAATTGGCTGGCCTGCGRPArsmARSDMRGAATGAAGGCGGATTAGCGCGGCGGCGGGAGGAGAGGAG			IF Mutant
ISINZADAINOCGACIC ITAGAGAGCGAAACCOGCAACATCCMutantrsmZSOEFGTTTTCTGGAGGTTGGGGAATTCCCGCCGCTTTTACTCGTCMutantrsmZSNTHFGCGCGCGCCGCACCGATCCAGGCGACGTCGAACTGCMutantrsmZIntRCATGCAGGAATTCATCGAGCTGMutantrsmYKOFCGCGGAGCTCCTGTTCACTCGAAGCGCAACCTCCAGMutantrsmYKOFCGCGGACTCTAGATTCGCCAACTCCGCGCCGAGGAAAACCGCGGTCGMutantrsmYSOEFCGCGTATAGATTCGCCAACTCCGCGCCGAGGAAAACCGCGGTCGMutantrsmYSOERCGACGCGGTTTTCCTCGGGCCGCAGGATCCTGACGGTTTGAAGATTACGCMutantrsmYSNTHFCTGGAACTGGAACGGCGAGMutantrsmYNTRGGAATTCAGGAAGGTGCCCMutantrsmYNTRGGCAGCTTACACTTTATGCTGCGGCCGCAGAGAAACCGGGTGGMutantrsmYintRGGCAGCTTACACTTTATGCTGCGGCTGGAATAATGTGGAGGLeader fusion vectorlacUVSNotlGGCCGCTTTACACTTATGCGAGGGAGTCGTCACCGTGCGGMutantrsmAnnealFGATCCTCCACACATTATACGAGCGGAAGACGCGGAGGGGGGGG	rsmZKpnIF		Mutant
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lacZRXholCCCCTCGAGCAGACATGGCCTGCCCGGTTATTATLF vectorlacZRforTFGATGTGCTGCAAGGCGATTAAGSEQrsmASDMAlgRFGAATTCGGCAGGAACTTTCATTCCGGCRPArsmARPARTAATACGACTCACTATAGGGAGAGATAAAAATTAATGGTTTGGCTCTTGRPArsmARSDMFCCTTTTGTCGTTTTTGAGAATTCGGCAGGAACTTTCATTCCGel shiftrsmARSDMRGAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGGGel shiftalgDBS1FCGCCTCCTGGCGCTACCGTTCGTCGCGCAGCGACGACGACGCGCGGel shiftalgDBS1RGCGACGCAGCAGGAGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCGGel shift	lacZScalF	GCTATGACCATGATTAGTACTGATTCACTGGCCGTC	TLF vector
lacZRforTFGATGTGCTGCAAGGCGATTAAGSEQrsmASDMAlgRFGAATTCGGCAGGAACTTTCATTCCGGCRPArsmARPARTAATACGACTCACTATAGGGAGAGATAAAAATTAATGGTTTGGCTCTTGRPArsmARSDMFCCTTTTGTCGTTTTTGAGAATTCGGCAGGAACTTTCATTCGel shiftrsmARSDMRGAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGGGel shiftalgDBS1FCGCCTCCTGGCGCTACCGTTCGTCCGCGACACCCCTGCGTCGCGGel shiftalgDBS1RGCGACGCAGCAGCAGGAGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCGGel shift	lacZRXhol	CCCCTCGAGCAGACATGGCCTGCCCGGTTATTA	TLF vector
rsmASDMAlgRFGAATTCGGCAGGAACTTTCATTCCGGCRPArsmARPARTAATACGACTCACTATAGGGAGAGATAAAAATTAATGGTTTGGCTCTTGRPArsmARSDMFCCTTTTGTCGTTTTTGAGAATTCGGCAGGAACTTTCATTCGel shiftrsmARSDMRGAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGGGel shiftalgDBS1FCGCCTCCTGGCGCTACCGTTCGTCGCGCAGCACCCCTGCGTCGCGel shiftalgDBS1RGCGACGCAGCAGCAGGAGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCGGel shift	lacZRforTF	GATGTGCTGCAAGGCGATTAAG	SEQ
rsmARPAR     TAATACGACTCACTATAGGGAGAGAAAAAATTAATGGTTTGGCTCTTG     RPA       rsmARSDMF     CCTTTTGTCGTTTTTGAGAATTCGGCAGGAACTTTCATTC     Gel shift       rsmARSDMR     GAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGG     Gel shift       algDBS1F     CGCCTCCTGGCGCTACCGTTCGTCGCGACACCCCTGCGTCGC     Gel shift       algDBS1R     GCGACGCAGCAGCAGGAGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCG     Gel shift	rsmASDMAlgRF	GAATTCGGCAGGAACTTTCATTCCGGC	RPA
rsmARSDMF     CCTTITGTCGTTTTTGAGAATTCGGCAGGAACTTTCATTC     Gel shift       rsmARSDMR     GAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGG     Gel shift       algDBS1F     CGCCTCCTGGCGCTACCGTTCGTCCCTCCGACACCCCTGCGTCGC     Gel shift       algDBS1R     GCGACGCAGCAGGAGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCG     Gel shift	rsmARPAR	TAATACGACTCACTATAGGGAGAGAGATAAAAATTAATGGTTTGGCTCTTG	RPA
rsmARSDMR GAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGG Gel shift algDBS1F CGCCTCCTGGCGCTACCGTTCGTCCCTCCGACACCCCTGCGTCGC Gel shift algDBS1R GCGACGCAGCAGGAGGAGGAGGAACGGTAGCGCCAGGAGGCG Gel shift	rsmARSDMF	CCTTTTGTCGTTTTTGAGAATTCGGCAGGAACTTTCATTC	Gel shift
algDBS1F     CGCCTCCTGGCGCTACCGTTCGTCCCTCCGACACCCCTGCGTCGC     Gel shift       algDBS1R     GCGACGCAGCAGGGGGTGTCGGAGGGGACGAACGGTAGCGCCAGGAGGCG     Gel shift	rsmARSDMR	GAATGAAAGTTCCTGCCGAATTCTCAAAAACGACAAAAGG	Gel shift
algDBS1R GCGACGCAGCAGGAGGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCG Gel shift	algDBS1F	CGCCTCCTGGCGCTACCGTTCGTCCCTCCGACACCCCTGCTGCGTCGC	Gel shift
	algDBS1R	GCGACGCAGCAGGGGTGTCGGAGGGACGAACGGTAGCGCCAGGAGGCG	Gel shift
rsmARBS1F GCGCCTTTGGCCGTTTGGCCGGCAGGAACTTCCG Gel shift	rsmARBS1F	GCGCCTTTGTCGTTTTGACCGTTGGCAGGAACTTCATCCG	Gel shift
rsmARBS1R CGGAA1GAAAGIICCIGGCAAACGGICAAAAGGCGC Gel shift	rsmARBS1R		Gel shift
pscrRBSF GCTGGCGGGGTGTGCCGGGGAGCTGGCCGGGGGGG Gel shift	pscFRBSF		Gel shift
pscrKBSR CCTCTGGCCAG1TCCCGGCAGCAC Gel shift	pscFRBSR		Gel shift
rsmAPE1F GCGCGCTACCAAGGATCCGCGCTATTCTGCGGATCCGCCGCCATTCTT Gel shift	rsmape i F		Gel shift
ISHIARESE GLAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG	ISIIIAPESE		Gel shift
ISIMAPESK GEGEGGATECICAECEAGIATIGAECEAGICE Gel snit	rsmapesk		Gel shift
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rsmAGSWTannealR GAAAGTTCCTGCCAAACGGTCAAAAACGACAAAAGGCGCGG Gel shift	rsmAGSWTannealR	GAAAGTTCCTGCCAAACGGTCAAAAACGACAAAAGGCGCGG	Gel shift

 $^{a}$ TF, transcriptional fusion; TLF, translational fusion; SEQ, sequencing primer; RPA, RNase protection assay.

Sequenase 7-deaza-deoxy-GTP (7-deaza-dGTP) kit (Affymetrix, Cleveland, OH). Both primer extensions and sequencing reactions were run on a 6% acrylamide-8 M urea gel. The gel was dried and extension products detected using autoradiography.

In vitro transcription and RPA. The rsmA, rsmY, and rsmZ probes for RNase protection assay (RPA) were synthesized using a PCR product generated with primers that incorporated a T7 promoter sequence at the 5' end and using the MAXIscript T7 kit (Life Technologies) (Table 1). The reverse primer containing the T7 promoter sequence contained a nonhomologous sequence to discriminate between the fulllength probe and protected fragments. The primers rsmASDMAlgRF and rsmARPAR were used to produce the PCR product for rsmA. The rsmYRPAT&R/rsmYRPAF primer pair for rsmY or rsmZRPAT7RPAF/ rsmZRPAF primer pair for rsmZ was used to produce probes for the appropriate target. Probes were labeled by biotin-11-UTP (Life Technologies) at a ratio of 4:6 with UTP and were gel purified after in vitro transcription. Five micrograms of total RNA from each P. aeruginosa strain was precipitated with 800 pg of labeled probe and resuspended in hybridization buffer [80% formamide, 400 mM NaCl, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA], heated 95°C for 5 min, and incubated overnight at 42°C. Negative controls using the probe incubated with 10  $\mu$ g of yeast RNA were also included. Digestion of unhybridized RNA was performed using RNase A at 25  $\mu$ g/ml and RNase T1 at 10 U/ml. A biotinylated RNA ladder (Kerafast) was also used to determine the size of protected probe fragments. The reactions were run on a 5% acrylamide-8 M urea gel for rsmA and a 10% acrylamide-8 M urea gel for rsmY and rsmZ. RNA was transferred to a positively charged membrane (Hybond N+; Amersham Biosciences) using a semidry blotting system and 0.5 imes Tris-borate-EDTA (TBE; Hoefer, Holliston, MA). Nucleic acid was UV cross-linked and probes detected using the chemiluminescent nucleic acid detection module (Thermo Scientific). After washing, the blot was incubated for 5 min and developed using a FluorChem M (ProteinSimple). RNase protection assays were performed at least three times.

**Northern blot analysis.** Total RNA was extracted from strains using the PureLink RNA minikit (Thermo Fisher). RNA was quantitated using a NanoDrop (Thermo Fisher). Two to 5  $\mu$ g of total RNA was electrophoresed through a 10% acrylamide-8 M urea gel using formaldehyde loading buffer. After electrophoresis, RNA was transferred using a semidry blotter (Hoefer, Holliston, MA) at 300 mAmp for 45 min and the membranes cross-linked using UV light (Bio-Rad). Probes produced by *in vitro* transcription were biotin labeled and hybridized to the membrane using ULTRAhyb buffer (Thermo Fisher) at 65°C overnight. The primers used to make probes for *rsmY* and *rsmZ* are listed in Table 2. For *rsmY*, the rsmYRPAF/rsmYT7RPAR primer pair was used. For *rsmZ*, the rsmZRPAF/rsmZRPART7 primer pair was used. Membranes were washed with two 5-min low-stringency washes at room temperature and 2 high-stringency washes for 15 min at 65°C. Blots were developed using the chemiluminescence detection kit (Thermo Fisher), in accordance with the manufacturer's instructions. For normalization, 55 RNA probes (Table 2) made by *in vitro* transcription were used using primers 55rRNAI and 55rRNA2T7. Northern blotting was repeated more than three times.

**Statistical analyses.** Statistical analyses were performed using Prism 6.0 (GraphPad software, La Jolla, CA). Transcriptional and translational fusions and densitometry analysis were compared using a one-way analysis of variance (ANOVA) with Tukey's posttest.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB .00048-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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